The molecular dialogue between *Eucalyptus grandis* and the myrtle rust pathogen, *Austropuccinia psidii*

by

Shae Swanepoel

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

In the Faculty of Natural & Agricultural Sciences

Department of Biochemistry, Genetics and Microbiology

University of Pretoria

Pretoria

20 December 2021

Supervisor: Prof. Sanushka Naidoo

Co-Supervisor: Dr. Louise S. Shuey

Declaration

I, Shae Swanepoel, declare this thesis, which I hereby submit for the degree M.Sc. 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.

Shae Swanepoel
20 December 2021

This dissertation is dedicated to my partner:

Mr. M. J. Cooper

who has supported me throughout my academic journey, constantly encouraged my curiosity and always pushed me further than I could have imagined.

Thesis Summary

Title: The molecular dialogue between *Eucalyptus grandis* and the myrtle rust pathogen, *Austropuccinia psidii*

Name: Shae Swanepoel

Supervisor: Prof. Sanushka Naidoo (University of Pretoria, South Africa)

Co-supervisors: Dr Louise S. Shuey (Department of Agriculture and Fisheries, Queensland Government, Australia)

Department: Biochemistry, Genetics and Microbiology, University of Pretoria

Degree: *Magister Scientiae*

Eucalyptus. are among the most important agricultural forestry species grown worldwide for their value in the paper, pulp, and timber industries. *E. grandis* is susceptible to the biotrophic rust pathogen, *Austropuccinia psidii*. This pathogen causes myrtle rust disease on growing leaves and shoots of many members of the Myrtaceae family. In *E. grandis*, symptoms of myrtle rust disease are variable, ranging from highly susceptible to resistant. These symptoms include light chlorotic flecking, evidence of hypersensitive responses, necrotic lesions and formation of severe pustules on the leaves and petioles. Despite the worldwide significance of myrtle rust, little is understood about the molecular dialogue that exists between host and pathogen. Unravelling the mechanisms governing this interaction will facilitate the development of robust disease management strategies. The aim of this study was to investigate the molecular dialogue between resistant and susceptible *E. grandis* and *A. psidii*, using a dual RNA-seq approach. *E. grandis* seedlings were collected from their natural range across the east coast of Australia and screened for resistance and susceptibility against the pandemic biotype of *A. psidii*. Leaf samples were collected at four time points, namely 12-hours post inoculation (hpi), 1-day post inoculation (dpi), 2-dpi and 5-dpi. RNA was isolated from these samples and subjected to RNA-seq using Illumina 50bp PE reads to a depth of 40 million reads per sample. RNA-seq reads were mapped to the *E. grandis* and *A. psidii* reference genomes to quantify expressed genes. In *E. grandis*, the resistant seedlings were able to maintain a controlled and coordinated response against *A. psidii*

throughout the interaction, with a hypersensitive response enriched at 1- and 2-dpi. In contrast, susceptible seedlings lacked a coordinated response, with absence of a hypersensitive response during crucial initial stages of infection. Brassinosteroid mediated signalling was enriched in resistant hosts at 2-dpi, with this response not observed in the susceptible hosts. Additionally, brassinosteroid signalling genes were found within two disease resistance loci. Numerous resistance genes were found underlying major disease resistance loci, with these genes differentially expressed at both the constitute and induced levels. These resistance genes are highlighted for future functional studies to determine the role they play during the interaction. These results will contribute to informed selective breeding to generate myrtle rust resistant plants. On the pathogen side, we identified a total of 890 expressed genes across the time series. Comparisons of the expressed genes to the candidate list of effector proteins found various expressed candidate effectors, including a rust transferred protein uniquely expressed in the susceptible hosts at 5-dpi. This protein causes the accumulation of chloroplasts around the haustorium, which may affect the functioning of the chloroplast. Functional enrichment identified malate dehydrogenase, malate metabolism and the involvement of oxoacids, putatively implicating oxalic acid in the interaction with susceptible hosts. The results from this study identifies numerous virulence and pathogenicity factors that can be targeted for disease control strategies. Future studies are required to determine the role brassinosteroids play in resistance, as well as investigating the involvement of oxalic acid in disease susceptibility.

Table of Contents

Preface

The aim of the thesis was to determine the molecular mechanisms that govern the interaction between *Austropuccinia psidii* and *Eucalyptus grandis* at four time points. With this knowledge, the differences in responses between resistant and susceptible hosts can be compared to determine what contributes to host resistance and pathogen success in susceptible hosts. This will further contribute to our understanding of the complex interaction between *A. psidii* and *E. grandis* to facilitate genetic engineering to breed *A. psidii* resistant plants.

Chapter 1 is a comprehensive review of our current knowledge about the myrtle rust fungus, *A. psidii* and the devastating impact this pathogen has on our natural and commercial forests. Furthermore, the significance of *Eucalyptus* and typical defence responses against biotrophic fungal pathogens are discussed.

Chapter 2 is a research chapter investigating the molecular responses of resistant and susceptible *E. grandis* during the interactions with *A. psidii* to identify novel pathways and mechanisms governing the different host responses. This is through the investigation of transcriptomic data obtained by a robust dual RNA-sequencing analysis.

Chapter 3 is a research chapter investigating the transcriptomic responses of *A. psidii* during the interactions with resistant and susceptible *E. grandis* over a time series. This is through the analysis of pathogen-specific gene expression to identify pathogenicity and virulence factors involved in the interaction.

Chapter 4, a concluding chapter, highlights the importance of this research, where a hypothesised model of the molecular dialogue is suggested. Limitations to the study are discussed, with future studies suggested to address these limitations.

Research outputs:

Research published in ISI rated journals:

Swanepoel S., Oates C. N., Shuey L. S., Pegg G. S., Naidoo, S. (2021). Transcriptome analysis of *Eucalyptus grandis* implicates brassinosteroid signalling in defence against myrtle rust (*Austropuccinia psidii*). *Frontiers in Forests and Global Change* 4,778611. doi: 10.3389/ffgc.2021.778611.

Research presented at international conferences:

Swanepoel S., Oates C. N., Shuey L. S., Pegg G. S., Naidoo, S. Brassinosteroid signalling underlies myrtle rust (*Austropuccinia psidii*) resistance in *Eucalyptus grandis*. Plant Biology 2021 Worldwide Summit. 19-23 July 2021. Virtual Conference. (Poster presentation).

Abbreviations

- •OH, hydroxyl radicals
- ABA, abscisic acid
- *ActA,* actin protein
- ATP, adenosine triphosphate
- AtPDI-11, *Arabidopsis* protein disulfide isomerase-11
- Avr, avirulence
- BAK1, BRI1-associated kinase 1
- BES1, BRI1-EMS-suppressor 1
- BGI, Beijing Genomics Institute
- BH, Benjamini and Hochberg
- BIK1, Botrytis-induced kinase 1
- BIN2, NR-insensitive 2
- BKI1, BR kinase inhibitor 1
- BL, brassinolide
- BLAST, Basic Local Alignment Search Tool
- BP, biological process
- BR, brassinosteroid
- BRI1, BR-insensitive 1
- BRZ1, brassinazole-resistance 1
- BSK1, BRI1-interacting signalling kinase 1
- BSU1, BRI1-suppressor 1
- Ca^{2+}, calcium
- Cac1, adenylyl cyclase
- cAMP, cyclic adenosine monophosphate
- CC, cellular component
- CDG1, constitutive differential growth 1
- CK, cytokinin
- COI1, coronatine insensitive 1
- *CPA1,* cyclophilin
- CRB BLAST, Conditional Reciprocal Best BLAST
- CWDE, cell wall degrading enzyme
- DE, differential expression
- DEGs, differentially expressed genes
- Dpi, days post inoculation
- EDS1, enhanced disease susceptibility 1
- EIN3, ethylene insensitive 3
- ERF, ethylene response factor
- ET, ethylene
- ETI, effector-triggered immunity
- ETS, effector-triggered susceptibility
- FABI, Forestry and Agricultural Biotechnology Institute
- FDR, false discovery rate
- FPKM, fragments per kilobase of transcript per million mapped reads
- GA, gibberellins
- GO, gene ontology
- H_2O_2, hydrogen peroxide
- HIGS, host-induced gene silencing
- Hpi, hours post inoculation
- HR, hypersensitive response
- HSPs, heat shock proteins
- ICS, isochromatic synthase
- ISR, induced systemic resistance
- JA, jasmonic acid
- JAZ, jasmonate-jim-domain
- JIN1, jasmonate insensitive 1
- KEGG, Kyoto Encyclopaedia of Genes and Genomes
- LRR, leucine-rich repeat
- Lys, lysine
- LysM, lysine motif
- MAMPs/PAMPs, microbial- or pathogen-associated molecular patterns
- MAPK, mitogen-activated protein kinase
- MeJA, methyl jasmonate

- MF, molecular function
- Mlp, *Melampsora larici-populina*
- NB-LRR, nucleotide binding and leucine-rich repeat domains
- NCBI, National Centre for Biotechnology Information
- NDR1, non-race specific disease resistance 1
- NO, nitrogen oxide
- NPR1, non-expression of *PR* genes 1
- NRF, National Research Foundation
- O_2^-, superoxide
- PAD4, phytoalexin deficient 4
- PAL, phenylalanine ammonia lyase
- *PdxS,* pyridoxal 5'-phosphate synthase
- PHI, pathogen-host interaction database
- PIGS, *in planta*-induced genes
- PK-LRR, protein kinase leucine-rich receptors
- PLP, pyridoxal 5'-phosphate
- PNPi, *Puccinia* NPR1 interactor
- PPR, pattern recognition receptors
- *Ppr, Puccinia psidii* resistance loci
- PR, pathogenesis-related
- PTI, PAMP-triggered immunity
- QTLs, quantitative trait loci
- *R*-genes, resistance genes
- R-interaction, resistant *Eucalyptus grandis*
- RNA-seq, RNA sequencing
- ROS, reactive oxygen species
- RTP1, rust transferred protein 1
- *S10e,* small subunit ribosomal protein
- SA, salicylic acid
- SAGE, serial analysis of gene expression
- SAR, systemic acquired resistance
- S-C vs. R-C, susceptible, control compared to resistant, control
- SERK1, somatic embryogenesis receptor-like protein kinase 1
- S-interaction, susceptible *Eucalyptus grandis*
- SM, secondary metabolism
- SNPs, single nucleotide polymorphisms
- SRA, short read archive
- SSPs, small, secreted proteins
- STAR, Spliced Transcript Alignment to a Reference
- TGA, TGA transcription factor
- TIR-NBS-LRR, toll/interleukin-1 receptor-NBS-LRR
- TPM, transcripts per kilobase million
- TPS2, trehalose 6-phosphate phosphatase
- *TUB1,* tubulin alpha-1 chain
- VST, variance stabilizing transformation
- WGCNA, weighted gene co-expression network analysis

Chapter 1

Literature Review

Elucidating the molecular interactions between *Eucalyptus grandis* and the myrtle rust pathogen, *Austropuccinia psidii*

Shae Swanepoel [1], Caryn N. Oates [1], Louise S. Shuey [2], Sanushka Naidoo [1]

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

[2] Department of Agriculture and Fisheries, Queensland Government, Australia

This chapter has been prepared in the format of a manuscript for a peer-reviewed research journal. I conceived the premise for this review with the input of Sanushka Naidoo. I drafted the manuscript and prepared the figures with the input of Caryn N. Oates, Louise S. Shuey and Sanushka Naidoo.

1.1 Abstract

Eucalypts are widely cultivated due to their significant wood quality and rapid growth, rendering them one of the most economically valuable forest plantation crops. *Eucalyptus* species are threatened by various emerging pests and pathogens, affecting the sustainability of our forest plantations while simultaneously posing a threat to the biodiversity of their natural range. *Eucalyptus grandis* is highly susceptible to *Austropuccinia psidii*, a biotrophic rust pathogen causing myrtle rust on approximately 480 species within 86 genera of the Myrtaceae family. With a worldwide distribution and broad host range, this pathogen is considered a global pandemic and extensive research has been conducted to elucidate the molecular mechanisms that govern the interaction between this pathogen and its hosts. The constitutive overexpression of resistance-related genes in *Eucalyptus grandis* are shown to play significant roles in resistance against *A. psidii*. However, despite recent studies on this interaction, further investigations into *E. grandis* host responses and *in planta A. psidii* pathogenic responses are required to unravel the molecular dialogue that exists in this pathosystem. Understanding these molecular interactions may lead to improved breeding strategies and engineering of resistance against *A. psidii*.

Keywords: *Eucalyptus*, RNA-seq, defence response, *R*-genes, myrtle rust

1.2 Introduction

Eucalyptus (Myrtaceae) are a large genus comprising approximately 700 species and hybrids. These fast growing, woody, dicotyledonous forest trees hold significant ecological and economic importance. They are cultivated worldwide due to their significant contributions to the paper, pulp and timber industries as well as providing resources to produce bioenergy and biomaterials (Grattapaglia et al., 2012; Myburg et al., 2014). With the threat of climate change as well as increased anthropogenic activities, the sustainability of forests across the globe are at risk (Wingfield et al., 2015). These activities drive increased emergence of pests and pathogens that pose devastating threats to our natural and cultivated forests. Introduced pathogenic species may undergo host shifts, affecting naïve plantations and are often without natural enemies (Slippers et al., 2005). This is a major concern as naïve species often do not have adequate defence mechanisms, placing these at risk of devastating losses and in extreme cases, extinction.

Recently termed one of the Top Ten most feared fungi (Hyde et al., 2018), *Austropuccinia psidii* (Winter) Beenken (Beenken, 2017) has fast become one of the most devastating fungal pathogens to plague our natural and introduced forest plantations. This pathogen, commonly referred to as myrtle rust, has a widespread global distribution, affecting numerous tree species within Myrtaceae and posing a threat to the biodiversity of natural populations as well as devastating economic losses in the forestry industry (Wingfield et al., 2008; Wingfield et al., 2015). *Eucalyptus* spp. are some of the most susceptible members of this family to *A. psidii* (Coutinho et al., 1998; Junghans et al., 2003). This highlights the importance of further studies into the mechanisms governing the interaction.

With improving next-generation technologies, we can gain holistic views of previously poorly understood interactions through investigations of changes in the transcriptomes of organisms of interest (Mortazavi et al., 2008). The availability of the *E. grandis* reference genome (Myburg et al., 2014) coupled with these improved techniques facilitates powerful and robust studies on the molecular mechanisms that underlie the interactions between pests and pathogens and *E. grandis.* It is through these studies that we gain insights into mechanisms that contribute to disease resistance, contributing to the development of disease tolerant hybrids to mitigate the effects of devastating pests and pathogens. This review highlights the significance of *Eucalyptus* spp., the complex biology of *A. psidii* and common plant defences against pests and pathogens. Where appropriate, known defence mechanisms of Myrtaceae against *A. psidii* are discussed. Biotrophic pathogenicity and virulence mechanisms are discussed, with comparisons made to *A. psidii* to elucidate putative molecular responses of myrtle rust. Furthermore, the current understanding of the interactions between *A. psidii*

and its hosts facilitated by high-throughput RNA-seq are discussed to shed light on this complex and significant pathosystem.

1.3 The genus *Eucalyptus*

Myrtaceae are a large family of dicotyledonous hardwood plants that typically occur in the Southern Hemisphere, found in sub-tropical and tropical regions of countries such as South America, Australia, Asia, Africa and Europe (Grattapaglia et al., 2012). This family is made up of economically and ecologically significant hardwood genera including *Eucalyptus, Corymbia, Melaleuca* and *Psidium* (Grattapaglia et al., 2012). Eucalypts are a diverse species within this family, comprising *Eucalyptus* L'Hérit., *Corymbia* (Hill and Johnson, 1995) and *Angophora* Cav. (Ladiges, 1997). Due to natural hybridisation, *Eucalyptus* species display high diversity, with members showing environmental adaptability and phenotypic plasticity, growing under various conditions (Grattapaglia et al., 2012), including climates ranging from tropical to temperate and in soil of poor quality and nutrients (Shepherd et al., 2011). This versatility, along with its significant wood quality and rapid growth contribute to its use in the forestry industry, providing invaluable resources for timber, paper and pulp (Grattapaglia, 2008), with an estimated over 20 million hectares cultivated worldwide (Iglesias-Trabado and Wilstermann, 2008). Moreover, *Eucalyptus* spp. hold significant ecological value, providing key resources among their natural range. Most eucalypts are native to Australia, where they make up 101 million hectares of forests across the country (Australia's state of the forests report 2018). These species support the native forest ecosystem by providing habitats and food for forest fauna species as well as supporting other forest flora species (Australia's state of the forests report 2018).

In recent years, many studies have focused on the genetic factors controlling economically important traits in eucalypts, such as the mechanisms underlying wood formation and lignin biosynthesis (Grattapaglia et al., 2012). These studies have been vastly facilitated by the release of the *E. grandis* (640-megabase, 36,376 predicted protein-coding genes) reference genome (Myburg et al., 2014). This resource has been invaluable in genomic and transcriptomic studies in *E. grandis.* Unsurprisingly, due to their widespread cultivation, *Eucalyptus* spp. have become targets for various emerging pests and pathogens (Wingfield et al., 2013). Therefore, focus is not only on economically important traits but also the molecular mechanisms that govern the interactions with invasive organisms to gain knowledge on biological pathways and key genes contributing to plant defence (Naidoo et al., 2014; Christie et al., 2015; Mangwanda et al., 2015; Oates et al., 2015; Meyer et al., 2016; Messal et al., 2019; Santos et al., 2020; Hsieh et al., 2021; Yong et al., 2021).

1.4 *Austropuccinia psidii*

Austropuccinia psidii, commonly referred to as eucalyptus, guava, or myrtle rust, is an obligate biotrophic rust pathogen causing rust disease on several genera of Myrtaceae. Formerly known as *Puccinia psidii*, this pathogen has been resolved in the Pucciniales, moving from Pucciniaceae to Sphaerophragmiaceae (McTaggart et al., 2016b; Beenken, 2017). *A. psidii* is not closely related to *Puccinia* but more closely related to *Dasyspora, Puccorchidium, Sphenorchidium* and *Sphaerophragmium*. Despite showing morphological similarities to the genus *Puccinia, A. psidii* is genetically different and thus the pathogen was reclassified (Beenken, 2017). *A. psidii* is thought to have originated in Central and South America, where it was first reported by Winter (1884) on guava (*Psidium guajava*). Since its discovery, it has been reported in many countries across the world, where it is affecting native and non-native Myrtaceae species, causing widespread devastation. Reportedly affected areas include numerous countries in Central and South America (Coutinho et al., 1998) and North America, including California, Florida and Hawaii in the USA (Marlatt and Kimbrough, 1979; Rayachhetry et al., 1997; Uchida et al., 2006). Reports of affected Asian countries include Japan (Kawanishi et al., 2009), China (Zhuang and Wei, 2011), Indonesia (McTaggart et al., 2016a) and Singapore (du Plessis et al., 2017). South Africa is the only African country to have reported the presence of *A. psidii* (Roux et al., 2016). Oceania countries reporting the presence of *A. psidii* include Australia (Carnegie et al., 2010), New Caledonia (Giblin, 2013) and New Zealand (du Plessis et al., 2019). Figure 1.1 summarises the global distribution of *A. psidii* at the country level.

A high level of diversity exists within *A. psidii*, with cross-inoculation studies revealing differences in pathogenicity of isolates sampled from different Myrtaceous hosts. There are at least four biotypes of *A. psidii*, each with varying degrees of pathogenicity and each biotype capable of infecting different hosts (Ross-Davis et al., 2014; Stewart et al., 2018). A study by Stewart et al. (2018) investigated the variation between and within *A. psidii* populations in the Americas and Hawaii. They found that isolates clustered into nine genetic clusters (C1-C9) with C1 and C4 classified as the devastating "Pandemic biotype". Currently, the "Pandemic biotype" is found in numerous countries across the globe, including parts of North America, Asia, Australia, Columbia and pacific countries such as New Zealand (du Plessis et al., 2019). Interestingly, a unique biotype of *A. psidii* was identified on a native Myrtaceous shrub in South Africa (Roux et al., 2016), with effects of this pathogen not as significant to that of the "Pandemic biotype".

Although this pathogen is not currently posing a threat in South Africa, it is expected that it may cause future devastation as the climate of the country changes. Roux et al. (2015) investigated the risk of

myrtle rust establishment in South Africa, where *Eucalyptus* spp. commonly cultivated in the forestry industry were screened for susceptibility to the "Pandemic biotype" of *A. psidii*. The authors found that coastal regions, which exhibit wet and humid conditions, are the most at risk for establishment of myrtle rust, particularly in the warmer summer months (Roux et al., 2015). This study showed that *Eucalyptus* species grown in the South African forestry industry are at risk of infection, with *E. urophylla* displaying high levels of resistance and *E. camaldulensis* showing high levels of susceptibility (Roux et al., 2015). The results of this study emphasise the importance of adequate screening protocols for *A. psidii* incursion in South Africa and the need for future studies that investigate the molecular mechanisms underlying the host responses of the commonly cultivated Myrtaceae species. This will aid in the improvement of selective breeding and engineering of plants that are tolerant and resistant to myrtle rust infection.

Recently, it has been shown that *A. psidii* is able to undergo sexual reproduction, a phenomenon that was previously poorly characterised (McTaggart et al., 2020). This, coupled with the presence of several biotypes, may facilitate increased pathogenicity through novel virulence mechanisms in *A. psidii*. This raises threats of genetic recombination between different biotypes, which will produce new strains able to overcome host adaptations and circumvent current control strategies. For this reason, extreme caution must be taken to prevent further spread of myrtle rust.

A. psidii reportedly affects approximately 480 species within 69 genera of Myrtaceae (Soewarto et al., 2019a). The number of reportedly affected species has dramatically increased over the years, with only 33 reportedly affected species in 1998 (Coutinho et al., 1998). These include reports of a staggering 392 affected plant species in Australia, 72 in Brazil and 53 in New Caledonia, while South Africa has reportedly 14 affected species of Myrtaceae (Soewarto et al., 2019a). Affected hosts include valuable hardwood plants such as *Eucalyptus* spp. used in forestry plantations for wood production as well as other agriculturally significant plants such as allspice (*Pimenta* spp.), guava (*Psidium* spp.), rose apple (*Syzygium* spp.) and tea tree oils (*Melaleuca* spp.). Numerous affected plants play integral roles as ecosystem drivers and the threat of species extinction by *A. psidii* may potentially alter ecosystem functioning and reduction in species biodiversity (Fensham et al., 2020). For example, *A. psidii* has swept through the Hawaiian Islands and posed a significant threat to various native species, such as ohia, *Metrosideros polymorpha* (Uchida et al., 2006). This species is crucial to the ecosystem functioning of Hawaiian forests, playing roles in shaping the structure of forest landscapes. *A. psidii* threatens this species and will have devastating impacts should repeated infections occur (Loope, 2010). In Australia, a native wild guava shrub *(Rhodomyrtus psidioides*) plays integral roles in the ecosystem by acting as a fire retardant. The spread of *A. psidii* across the country has brought this

species to near extinction and as result, there are increased risks of outbreaks of wildfires (Fensham et al., 2020). The rapidly expanding host list, the threat to economically and environmentally significant species as well as the identification of biotypes that can undergo sexual reproduction highlights the dangers of this pathogen. This reiterates the growing need of developing robust control strategies and surveillance techniques to mitigate its effects.

1.4.1 Biology and symptoms of infection

Obligate biotrophic microorganisms invade living plant tissues to exploit their hosts for nutrients, which aids in pathogen reproduction, proliferation and completion of their complex life cycles (Lo Presti et al., 2015). These pathogens include powdery mildews, smut fungi and rust fungi. *A. psidii* has a macrocyclic, obligate biotrophic lifecycle in which there are four known life stages, namely aecia, uredinia, telia and basidia (Glen et al., 2007; Morin et al., 2014). *A psidii* is an autoecious pathogen that completes its lifecycle on a single host (Glen et al., 2007; McTaggart et al., 2018). McTaggart et al. (2018) showed that basidiospores are produced on the same host that telia and uredinia are formed, which rules out the heteroecious hypothesis. This confirms that *A. psidii* does not have an aecial life stage. Figure 1.2 summarises the known lifecycle of *A. psidii*.

Completing its lifecycle on a single host allows the rapid spread of *A. psidii*, contributing to the widespread distribution and pathogenicity. Urediniospores are produced during the uredinia life stage (stage II, Figure 1.2). Many factors influence the germination of urediniospores, with light intensity, leaf moisture and atmospheric temperatures significant contributors to spore success (Glen et al., 2007). In favourable conditions, windborne urediniospores will germinate on the plant surface, upon which the appressorium develops. These appressoria develop from the tip of the germ tube which results in an infection peg. A recent study by Yong et al. (2019) reported non-stomatal penetration by *A. psidii*. Infection pegs directly penetrate the leaf cuticle and epidermal cell walls, entering the mesophyll cells. This characteristic of *A. psidii* is unusual, as urediniospores of rust fungi generally penetrate host plants through the stomata. This may explain the broad host range of *A. psidii* as penetration sites are less specific and locating the stomata is not necessary, which aids in a simpler infection process (Adendorff and Rijkenberg, 2000). Xavier et al. (2015) found reduced fungal germination, appressorium formation and penetration as the leaf matured, suggesting younger leaves have a higher susceptibility to *A. psidii*. Mature leaves have greater numbers of cuticle waxes that alters the leaf topography. This change alters the ability of spores to successfully identify and adhere to the plant surface, reducing *A. psidii* pathogenicity (Xavier et al., 2015). However, on susceptible hosts that *A. psidii* can successfully penetrate, the fungus moves intercellularly through the host tissues,

where mycelial growth has been reported. Subsequently, haustorium mother cells form from the hyphae (Coutinho et al., 1998). In *E. grandis*, haustoria are reported to begin developing between 12 and 18 h in susceptible genotypes and between 18 and 24 h in resistant genotypes (Xavier et al., 2001). It is through these haustoria that *A. psidii* obtains nutrients for successful growth and survival. Furthermore, haustoria are involved in suppressing host defences and reprogramming of host responses, contributing to pathogenicity of the fungus (Voegele and Mendgen, 2003).

A. psidii primarily affects young Myrtaceae seedlings less than two years old, nursery seedlings and young coppice, causing considerable damage to plant leaves and shoots. This may ultimately lead to the death of the plant (Glen et al., 2007). Generally, symptoms are variable between hosts (Minchinton et al., 2014). For example, Carnegie et al. (2010), reporting the first case of *A. psidii* in Australia, describes disease symptoms as yellow uredinial pustules on both the adaxial and abaxial surfaces of *Agonis flexuosa* leaves. These bright yellow pustules were seen on young stems and growing shoots and severely infected plants resulted in shoot death. On *S. glomulifera,* early infection symptoms presented as small purple flecks on young leaves which later resulted in the characteristic yellow pustules (Carnegie et al., 2010). Disease symptoms on *Metrosideros polymorpha* in Japan were first described as red spots on shoots and surfaces of plant leaves. These red spots develop into yellow uredinial sori, which increased in size before production of urediniospores (Kawanishi et al., 2009). In *Eucalyptus,* considered highly susceptible to *A. psidii* (Coutinho et al., 1998), symptoms typically present in a range from small uredinia to masses of bright yellow urediniospores, with lesions darkening on leaf and shoot margins (Glen et al., 2007).

Although symptoms vary between different hosts, generally young seedlings show visible symptoms within a few days of inoculation, characterised by yellow spots on leaves and shoots (Coutinho et al., 1998). After approximately two weeks following inoculation, brown lesions with bright yellow masses develop on leaves (Figure 1.3), occasionally shoots, fruits and flowers (Coutinho et al., 1998; Glen et al., 2007; Pegg et al., 2014). Disease severity varies between individual trees, with some only showing minor symptoms, while others present with reduced fecundity, loss of flowers and fruits and occasionally tree mortality (Pegg et al., 2014). Severe infection on susceptible hosts in plantations may lead to a reduction of their market value in the commercial industry, as growth is stunted and trees grow multiple branches with affected leaves and fruits, which is considered economically undesirable (Booth et al., 2000). Additionally, severe infection of this pathogen in natural forests may cause widespread reductions in species, affecting the natural biodiversity and ecology (Glen et al., 2007).

1.4.2 Control and management strategies

Once established in an area, mitigation of *A. psidii* becomes increasingly difficult. This is due to the lifestyle of the pathogen, in which rapid sporulation and windborne urediniospores facilitates spread across large geographical ranges (Zauza et al., 2015). Prevention of rapid spread of fungal spores relies on stringent surveillance of susceptible forests and plantations, monitoring for outbreaks before they spread beyond the initial site followed by accelerated intervention to remove infected trees (Glen et al., 2007). Additionally, management strategies involve reducing the cultivation of susceptible hosts and restricting movement of host material by reducing sale of products (Villalta et al., 2018). Chemical treatments typically involve the use of fungicides to control the spread and proliferation of the pathogen. Triadimenol, a triazole systemic fungicide, has been found to be effective in controlling *A. psidii*, with reportedly 90% efficacy in *E. cloeziana* seedlings (Alfenas et al., 1993). Further studies found azoxystrobin, tebuconazole, epoxiconazole and pyraclostrobin as effective fungicides against *A. psidii* (reviewed in Glen *et al.* 2007). Despite the efficacy of these fungicides, spray intervals need to be repeated regularly and this is not economically practical. Moreover, these chemicals pose calamitous effects on the environment, with varying toxicity levels to aquatic and terrestrial organisms (reviewed in Glen *et al.* 2007). As a result, more economically and environmentally feasible control strategies are required.

Additional control strategies involve the use of biological control agents (Glen et al., 2007). In New Zealand, *Eriococcus coriaceus* infests *Eucalyptus* plantations, affecting the commercial forestry industry. Efforts to eradicate this pest involve the introduction of predators, shown to be an effective control (Garnas et al., 2012). *Botrytis cinerea* causes grey-mould disease in susceptible *E. grandis* that causes damage to eucalypt plantations. It has been shown that *E. grandis* plants pre-infected with *Streptomyces* have reduced symptoms when subsequently infected with *B. cinerea,* due to the production of defence compounds such as phenols and flavonoids (Salla et al., 2016). Elicitation of plant defences with the use of biological control agents is an alternative method of traditional chemical treatment. This is an avenue that can be explored to mitigate *A. psidii* infection. For example, *Fusarium decemcellulare* has been shown to parasitize *A. psidii* and *Bacillus subtilis* reduces *A. psidii in vitro* germination by as much as 34% (Amorim et al., 1993; Glen et al., 2007). *F. fujikuroi* and *F. solani* were also found as hyperparasites on the spores of *A. psidii* (Lira et al., 2019). Further studies are needed to determine if these effects are repeated in field trials to ensure efficacy and safety in using these agents as biological control.

These eradication efforts are unlikely to be effective against *A. psidii* over an extended period. These efforts may be started too late to prevent the rapid spread by air-borne urediniospores. For example, upon initial discovery in Australia in 2010, responses included surveillance of sites, quarantining affected host plants or removal and destruction of infected material (Carnegie and Cooper, 2011). Despite this, *A. psidii* continued to rapidly spread and focus was forced to shift from eradication to management (Villalta et al., 2018). Whilst biological control may be a prospective avenue for control of *A. psidii*, there are risks to implementing these agents. For example, these agents may act too slowly to mitigate the effects of *A. psidii* and due to the nature of this pathogen rapid action is required to prevent further spread (Roux et al., 2013). Additionally, if conditions are favourable, these biocontrol agents may become pathogenic, either to the host they are protecting or to surrounding plant species (Chandrashekara et al., 2012).

For these reasons, control against *A. psidii* has begun focusing on host resistance. This involves selectively breeding or genetically engineering resistant myrtle species for large-scale cultivation (Wingfield et al., 2013). Cultivating hybrid resistant genotypes with significant defence systems are highly effective mechanisms to mitigate the effects of *A. psidii* and may reduce the spread of the pathogen (Coutinho et al., 1998; Wingfield et al., 2015). To successfully breed or engineer rust resistance will be multifaceted. Integrating complex breeding systems to select the fittest genotypes against *A. psidii* can be implemented to improve genomic selection. A combination of omics will facilitate identification of novel pathways and genes involved in resistance (Naidoo et al., 2019). Transcriptomics is a powerful tool that studies the gene expression profiles of organisms under different conditions, with dual RNA-seq used to simultaneously capture both host and pathogen transcripts during interactive stages (Mortazavi et al., 2008; Westermann et al., 2012). Studies of this nature are important in highlighting candidate genes for functional studies. In combination with proteomics and genomics, these studies are powerful in unravelling complex host-pathogen molecular dialogues (Naidoo et al., 2019). This is discussed further in Section 1.7: Current knowledge on the *Austropuccinia psidii*-host molecular dialogue.

1.5 Plant defences

Due to the sessile nature of plants, exposure to pests and pathogens is inevitable. Resultantly, plants have evolved complex defence mechanisms that are mounted in response to invading organisms, including bacteria, fungi, viruses, and insects. Responses involve constitutive defence mechanisms including preformed and chemical barriers, rapid recognition of invading organisms that breach these barriers and induced defence mechanisms deployed by the plant once an invading organism is

recognised. In combination, these responses attempt to prevent proliferation and success of the invading organism to ensure the health of the plant.

1.5.1 Constitutive defence mechanisms

Constitutive defence mechanisms are the first line, nonspecific, broad-spectrum defence against invading organisms and include physical and chemical barriers (Kovalchuk et al., 2013). Physical barriers involve waxy leaf cuticles, trichomes, cell walls, and bark while chemical barriers include secretory cells and glands, secondary or specialised metabolites, and antimicrobial compounds (Naidoo et al., 2014).

In *Eucalyptus* spp. the cuticular waxes chemical composition are important preformed barriers providing defence against *A. psidii* (dos Santos et al., 2019). There is significant variation between the cuticular wax composition among the different *Eucalyptus* spp. in addition to variation within the same species. These differences may contribute to the differences in responses to *A. psidii*, highlighting the significance of the wax composition in resistance against this fungus. The cuticular waxes of susceptible *E. grandis* contains hexadecenoic acid that was found to improve the growth and germination of *A. psidii*, putatively contributing to disease susceptibility (dos Santos et al., 2019). Primary hyphae of *A. psidii* are observed at 12-hpi (hours post inoculation) in susceptible genotypes of *E. grandis,* while only observed in resistant genotypes at 18-hpi, suggesting resistant genotypes have effective preformed barriers to prevent early hyphal formation, which may contribute to the resistance observed (Xavier et al., 2001).

Plant specialised metabolites (also referred to as secondary metabolites, SM) are toxic, antimicrobial substances produced by the plant in response to various environmental stimuli and include compounds such as phenolics, terpenes, alkaloids, and flavonoids. Abiotic factors influencing the production of SMs include temperature, water conditions, and light intensity as well as soil composition and fertility (Yang et al., 2018; Isah, 2019). Pathogenic fungi and bacteria, viruses and herbivory are biotic stressors that stimulate the production of SMs, resulting in a degree of plant defence against these invading organisms (Khare et al., 2020; Mishra et al., 2020).

Essential oils, stored in mature leaf oil glands, have potent antimicrobial properties comprising a combination of monoterpenes and sesquiterpenes. These terpenoid compounds have various functions in plant defence, including deterrents of herbivores and pests, attracting enemies of pests and defence against fungal pathogens (Keszei et al., 2008). The terpenoid backbone biosynthesis pathway was found to be significantly over-expressed in Norway spruce (*Picea abies*) upon infection with

Chrysomyxa rhododendri, a fungal pathogen causing bladder rust (Trujillo-Moya et al., 2020). Genes associated with the mevalonate pathway were over-expressed, showing the putative involvement of the terpenoids in defence against this rust pathogen (Trujillo-Moya et al., 2020). In *E. grandis,* monoterpenes and sesquiterpenes were found to contribute to resistance against the devastating galling insect, *Leptocybe invasa* (Oates et al., 2015). In the resistant genotypes, monoterpene concentrations were significantly greater than in the susceptible genotypes. Furthermore, RNA-seq analysis identified up-regulation of the terpenoid biosynthetic process, corroborating the results of the chemical profiling (Oates et al., 2015). A recent study investigated the leaf chemistry of *Corymbia citriodora* in response to a native pathogen (*Q. pitereka*) and the exotic fungal pathogen, *A. psidii* (Bonora et al., 2020). The authors found that the monoterpene concentrations in leaves infected with *A. psidii* were significantly different to that infected with *Q. pitereka* suggesting that not only do Myrtaceae species initiate pathogen-specific responses, but that terpene concentrations may contribute to disease resistance against *A. psidii* (Bonora et al., 2020).

The chemical composition of leaves change as the plant matures, which owes to the resistant phenotypes we see as plants age, with younger plant material showing greater susceptibility to foliar pathogens. For example, a recent study by Silva et al. (2020b) investigated the chemical composition of different leaf stages in resistant and susceptible *E. urophylla* × *E. grandis* hybrids against *A. psidii*. The authors found that the composition of the leaves at various stages varied, with no disease symptoms seen on resistant leaves at all stages, while no disease symptoms were observed on the last leaf stage in susceptible hybrids. Through gas chromatography and mass spectrometry, they found greater levels of limonene in resistant hybrids while there was little limonene in susceptible hybrids (Silva et al., 2020b). Limonene is an antifungal SM that has fungitoxic effects (Aggarwal et al., 2002). Silva et al. (2020b) found that limonene causes damage to the fungal cell walls while affecting the structures of teliospore, rendering the fungus non-pathogenic and unable to complete its life cycle effectively. In *M. quinquenervia,* chemotypic variation between resistant and susceptible individuals did not contribute to the disease outcome against *A. psidii*, but a susceptible chemotype was found to have higher levels of terpenes, suggesting that myrtle rust infection induces the production of certain SM and this potentially contributes to susceptibility (Hsieh et al., 2021).

The phenylpropanoid pathway produces numerous plant defence metabolites primarily in response to pathogenic fungal invasion (Kovalchuk et al., 2013). The derivatives of the phenylpropanoid pathway include phenols and lignin, responsible for the defence responses mounted against invasions. A recent study by Silva et al. (2020a) used mass spectrometry and a molecular networking approach to investigate the responses of Asian Soybean to the rust pathogen *Phakopsora pachyrhizi*. The authors

found production of various SM, including phenylpropanoids in plants infected with this pathogen with phenylpropanoids including liquiritigenin, coumestrol and formononetin among others (Silva et al., 2020a). Furthermore, in *Syzygium luehmannii*, the phenylpropanoid pathway was found to putatively confer resistance against *A. psidii*. Tobias et al. (2018) found up-regulation of phenylpropanoid pathway-associated genes in resistant genotypes, while susceptible genotypes showed comparatively weaker or absent associations with this pathway. Similarly, the involvement of the phenylpropanoid pathway was found in *E. grandis* through serial analysis of gene expression (SAGE). Differential expression of genes associated with lignin biosynthesis were identified in resistant genotypes, while susceptible genotypes lacked these responses (Moon et al., 2007). These studies demonstrate the importance of this metabolite in defence against rust pathogens, including *A. psidii*.

Constitutive defences, including physical and chemical barriers, are successful in preventing invasion by some pests and pathogens. However, compatible pathogens can overcome these defences and successfully penetrate and infect hosts. Therefore, it is important that plants have further defence mechanisms that are induced following breach of these preformed defences, which involves successful recognition of the invading organisms and deployment of appropriate defence responses (Jones and Dangl, 2006).

1.5.2 Induced resistance

The plant innate immune system involves a two-tier defence response (Jones and Dangl, 2006; Zipfel, 2014). The first branch of the innate immune system is triggered upon plant recognition of conserved, slowly evolving pathogen molecules, such as bacterial flagellin or fungal chitin (Nürnberger et al., 2004; Ausubel, 2005). These molecules are commonly referred to as microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs), respectively (Ausubel, 2005; Jones and Dangl, 2006; Couto and Zipfel, 2016). These molecules are recognised through transmembrane proteins known as pattern recognition receptors (PRR), with a leucine-rich repeat (LRR). PRRs recognise proteins such as bacterial flagellin and lysine motif (LysM) binding carbohydrates such as fungal chitin (Jones and Dangl, 2006; Zipfel, 2014). Once these MAMPs/PAMPs are recognised by the PRRs, a broad-spectrum, relatively weak immune response known as PAMP-triggered immunity (PTI) is induced, to prevent spread and proliferation against non-adapted pathogens (Jones and Dangl, 2006).

Adapted pathogens may successfully suppress PTI responses. This is through the secretion of pathogen effector proteins into the host plant cell, which contributes to pathogen virulence (Jones and Dangl, 2006). Intracellular effectors can reprogram the plant cell to allow the pathogen to acquire plant

nutrients, suppress host immunity and create an environment conducive to successful pathogen proliferation (discussed further in Section 1.6: Fungal pathogenicity and virulence mechanisms) (Lo Presti et al., 2015). This leads to effector-triggered susceptibility (ETS), in which the pathogen successfully overcomes plant defence, and the plant shows disease symptoms. Plants have evolved complex mechanisms to overcome ETS. This is through *R*-genes that can recognise pathogen effectors. *R*-genes produce proteins with nucleotide binding (NB) and leucine-rich repeat (LRR) domains (Jones and Dangl, 2006). NB-LRR R proteins are crucial to the innate immunity of plants. These proteins confer immunity by directly interacting with pathogen virulence factors or effectors, or indirectly through accessory proteins (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The accessory protein undergoes conformational changes upon interactions with the pathogen effector. The alteration of the accessory protein enables it to be recognised by the NB-LRR protein and through this, an ETI response is induced (van der Hoorn and Kamoun, 2008). This is known as the guard/decoy model (Dodds and Rathjen, 2010). Despite decades of research on R proteins and research elucidating the resistance these proteins confer, the mechanisms underlying R protein activation of ETI responses is poorly understood (Lai and Eulgem, 2018).

In general, ETI is more rapid than PTI and more effective at preventing spread of the invading pathogen. This is through the induction of the hypersensitive response (HR), a response that causes rapid, programmed cell death to prevent healthy neighbouring cells from infection (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Significant changes in plant gene expression induced by ETI responses leads to downstream activation of defence-related processes to confer resistance against the invading pathogen. The cycling between PTI, ETS and ETI leads to what is known as the zig-zag model of plant defence (Jones and Dangl, 2006). This model states that an invading pathogen initiates PTI through the recognition of MAMPs/PAMPs by plant PRRs. Following this, the pathogen evolves specific effectors to successfully overcome PTI, known as ETS. The plant may then evolve specific R proteins to mitigate the effect of the effectors, through the induction of robust HR that ultimately leads to ETI (Jones and Dangl, 2006).

There are a number of *R*-genes that have been highlighted as potential contributors to disease resistance against *A. psidii* in different Myrtaceae species. In *E. grandis*, numerous *R*-genes were found within the disease resistance locus *Ppr1* (Thumma et al., 2013), with these putatively contributing to the resistance of individuals carrying this loci. Furthermore, Swanepoel et al. (2021) showed many *R*-genes within the disease resistance loci, with expression induced upon infection with myrtle rust, suggesting a role in plant defence against *A. psidii*. Santos et al. (2020) identified *R*-genes differentially expressed at a constitutive level between resistant and susceptible *E. grandis*, with greater expression

in resistant genotypes. *R*-gene expression were also induced in *M. quinquenervia* and *S. luehmannii* upon infection with myrtle rust (Hsieh et al., 2018; Tobias et al., 2018). Future studies are required to characterise the function of these genes and to determine the roles they play during the interaction with myrtle rust.

1.5.3 Defence responses

The responses that occur following PTI and ETI is the rapid production and accumulation of cytosolic calcium (Ca^{2+}) (Boller and Felix, 2009). The increase of cytosolic Ca^{2+} functions to regulate extracellular reactive oxygen species (ROS) concentrations, including nitrogen oxide (NO), hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals (•OH) (Yu et al., 2017). The rapid production and accumulation of ROS is referred to as ROS burst and is vital in the first responses against pathogenic invasion (Torres et al., 2006). These molecules act as barriers to subsequent pathogenic invasion by strengthening plant cell walls as well as inducing HR (Yu et al., 2017). The importance of ROS bursts was demonstrated by Plotnikova et al. (2016). Timopheevii wheat (*Triticum timopheevii*) pre-treated with salicylic acid (SA), a phytohormone that induces ROS production and accumulation, was found to accumulate $O_2^{\bullet-}$. This led to the inhibition of brown rust fungus (*Puccinia triticina*) at the stomata and subsequent accumulation of H_2O_2. Plants that were treated with a ROS burst inhibiting chemical, verapamil, were more susceptible to brown rust fungus, with the suppression of oxidative burst facilitating pathogen proliferation (Plotnikova et al., 2016). Typically, these oxidative bursts of ROS occur in both resistant and susceptible plant species, with further responses occurring only in resistant plants to prevent spread of the pathogen (Low and Merida, 1996).

The rapid accumulation of cytosolic Ca^{2+} also leads to downstream transcriptional reprogramming to bring about immune responses, by activation of mitogen-activated protein kinase (MAPK) cascades (Boller and Felix, 2009). MAPK cascades lead to downstream activation of transcription factors and genes involved in plant defence (Dodds and Rathjen, 2010). Further downstream responses activated upon induction of PTI and ETI include plant phytohormone biosynthesis and signalling, stomatal closure, biosynthesis of antimicrobial compounds and callose deposition (reviewed in Yu et al. (2017)). Accumulation of Ca^{2+} also leads to the production of pathogenesis-related (PR) proteins, such as chitinases and thaumatin-like proteins, and there are 17 known PR families (van Loon et al., 2006). Induction of phytohormone signalling pathways, such as SA and jasmonic acid (JA), contributes to the differential expression of *PR* genes (Naidoo et al., 2013; Ali et al., 2018). This leads to an accumulation of these antimicrobial proteins that confer plant resistance against an invading pathogen (Pieterse et al., 2009).

Overall, these responses aim to prevent spread of the invading pathogen beyond the initial site of infection and prevent further proliferation of the pathogen. In general, various immune signalling and downstream defence responses are shared by PTI and ETI (Pitzschke et al., 2009). It is the strength and duration of these responses that differ, with ETI exhibiting robust, significant HR and oxidative burst while PTI is a comparatively weaker, broad-spectrum response (Dodds and Rathjen, 2010).

1.5.4 Phytohormones

Phytohormones are a group of signalling plant hormones that play integral roles in plant defences. Phytohormone biosynthesis signal transduction acts downstream of PTI and ETI once the plant has identified an invading organism (De Vos et al., 2005; Bari and Jones, 2009). Major defence-related hormones include SA, JA and ethylene (ET) (Pieterse et al., 2012). Other phytohormones that mediate plant growth, development and responses to abiotic stressors have emerged as important contributors to plant defence. These include abscisic acid (ABA), auxins, gibberellins (GA), cytokinin (CK) and brassinosteroids (BR) (Pieterse et al., 2009). SA is generally considered a phytohormone in defence against biotrophs, while JA/ET confer resistance against hemi-biotrophs and necrotrophs (Pieterse et al., 2009). Evidence has emerged over the years of a more complex interaction between phytohormones and the lifestyle of the invading pathogen, with an integrated phytohormone signalling system conferring resistance against invading organisms. This unconventional phytohormone signalling is seen in woody trees such as *Populus* and *Eucalyptus*, in which JA/ET conferred resistance against a biotroph and SA conferred resistance against a necrotroph, respectively (Azaiez et al., 2009; Naidoo et al., 2013). This review will cover typical defence phytohormones, including SA, JA/ET, and BR. For a comprehensive review on phytohormones modulating immunity, refer to Pieterse et al. (2012) and Bari and Jones (2009).

BRs are a group of steroidal phytohormones that primarily regulate plant growth and development (Grove et al., 1979; Saini et al., 2015). This group of phytohormones, responsible for regulating responses against abiotic stimuli, have been implicated in interactions with a range of pathogens, typically conferring resistance to biotrophs while increasing susceptibility to necrotrophs (Yu et al., 2018). In the absence of BRs, the receptors BR-INSENSITIVE 1 (BRI1) is associated with the negative regulators BR KINASE INHIBITOR 1 (BKI1) and BOTRYTIS-INDUCED KINASE 1 (BIK1), preventing its activation of downstream responses (Wang and Chory, 2006; Wang et al., 2008). In this state, the shaggy-like kinase BR-INSENSITIVE 2 (BIN2) is highly active, interacting with key BR transcriptional regulators BRASSINAZOLE-RESISTANT 1 (BRZ1) and BRI1-EMS-SUPPRESSOR 1 (BES1), inhibiting their function in BR signalling (He et al., 2002). In the presence of this

phytohormone, BR associates with BRI1 leading to phosphorylation and subsequent dissociation of BRI1 with its inhibiting regulators BKI1 and BIK1. This leads to the activation of BRI1-ASSOCIATED KINASE 1 (BAK1) and phosphorylation of BRI1-INTERACTING SIGNALLING KINASE (BSK1), activation of CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) and finally, BRI1-SUPPRESSOR 1 (BSU1) (De Bruyne et al., 2014; Yu et al., 2018). This cascade of events ultimately leads to the inactivation of BIN2, the activation of BRZ1 and BES1 and subsequent activation of downstream BR-specific responses through changes in gene expression (Tang et al., 2011).

The role that BRs play in plant defence are complex. This phytohormone has been shown to confer systemic resistance to the rice blast fungal disease (*Magnaporthe grisea*) and the bacterial blight disease (*Xanthomonas oryzae*) through exogenous applications (Nakashita et al., 2003). There are also reports of this phytohormone negatively regulating defence against pathogenic invasion. BRs can increase susceptibility to the viral infection caused by rice black-streaked dwarf virus (He et al., 2017), suggesting that the interaction with this phytohormone and pathogenic organisms is complex and integrated. Further research is needed to determine how this phytohormone confers resistance against some organisms while increasing susceptibility against others.

JA and its derivatives are part of a class of fatty acids that play roles in plant growth and development and are crucial in the plant defence arsenal against pests and pathogens (Wasternack and Hause, 2013; Ruan et al., 2019). This phytohormone is typically involved in resistance against necrotrophic pathogens and herbivores, such as insect pests (Wasternack and Hause, 2013). Transcription factors that are crucial to JA signalling in response to plant defence include JASMONATE INSENSITIVE 1/MYC2 (JIN1/MYC2) and ETHYLENE-RESPONSIVE FACTOR (ERF) (Verma et al., 2016). ERF proteins play a role in initiating the defence-related gene expression against necrotrophic pathogens (Moffat et al., 2012). In the absence of JA, JASMONATE-JIM-DOMAIN (JAZ) interacts with JIN/MYC2 to inhibit defence-related gene expression (Chini et al., 2007). When JA levels increase, it interacts with CORONATINE INSENSITIVE 1 (COI1) to lead to the degradation of JAZ and the subsequent up-regulation of associated genes (Chini et al., 2007).

ET is another phytohormone that interacts with JA to induce a defence response against pests and pathogens. An important ET transcription factor, ET INSENSITIVE 3 (EIN3) induces downstream expression of *ERF* genes to lead to defence responses in the presence of pests and pathogens (Verma et al., 2016). The interactions between JA and ET are vital in inducing defence-related responses upon pathogenic invasion (Glazebrook, 2005). JA and ET signalling are crucial in the development of

induced systemic resistance (ISR) due to root colonisation by rhizosphere bacteria. ISR primes the plant for defence against future pathogenic invasion, ultimately limiting plant disease severity (van Loon et al., 1998).

ISR is typically more effective against pests and necrotrophic fungi, however, there have been studies showing the importance of this resistance mechanism against certain biotrophic pathogens. Plants defective in efficient JA signalling are shown to be more susceptible to some biotrophic pathogens, suggesting an importance of this pathway in biotrophic defence mechanisms (Antico et al., 2012). For example, mutations in *COI1,* responsible for crucial JA signalling, rendered defective plants more susceptible to the biotrophic fungi, *G. cichoracearum* (Kloek et al., 2001). Furthermore, exogenous applications of methyl jasmonate (MeJA) to the conifer whitebark pine (*Pinus albicaulis*) triggered activation of downstream defence responses against the biotrophic fungal pathogen, white pine blister rust (*Cronartium ribicola*), putatively conferring resistance against this pathogen (Liu et al., 2017).

SA, a phenolic compound, is an important phytohormone in plant defence that is triggered upon recognition of pathogenic invasion, typically associated with defence against biotrophic and hemi-biotrophic pathogens (Glazebrook, 2005; Mishina and Zeier, 2007). SA is synthesised by two main pathways, namely PHENYLALANINE AMMONIA LYASE (PAL) and ISOCHORISMATE SYNTHASE (ICS) (Zhang and Li, 2019). The type of proteins that are involved in the production of SA are dependent on the type of pathogen recognition by the plant. Biosynthesis of SA in response to PAMP recognition involves ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) while biosynthesis of SA in response to effector recognition by R proteins involves EDS1, PAD4 and NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) (Reviewed in Pieterse et al. (2012)).

Following biosynthesis of SA, downstream signalling relies on a protein known as NON-EXPRESSOR OF *PR* GENES 1 (NPR1). Wu et al. (2012) found that NPR1 is the receptor for SA perception and that SA acts to activate NPR1 by inducing conformational changes upon binding to its domains. Subsequently NPR1 interacts with its associated transcription factors to bring about expression of *PR* genes, which leads to the production of antimicrobial proteins such as chitinases and peroxidases (Zhang et al., 2003; van Loon et al., 2006). SA signalling is also crucial to the establishment of broad-spectrum, long-lasting defence known as systemic acquired resistance (SAR), that is associated with the accumulation of PR proteins (Durrant and Dong, 2004; Mishina and Zeier, 2007).

Studies show the importance of SA in defence against biotrophic rust pathogens, both in herbaceous and woody plants. Crampton *et al.* (2009) reported reduced rust disease in pearl millet against the rust pathogen *P. substriata* upon exogenous application of SA, highlighting the importance of this phytohormone in defence against biotrophic pathogens. Interestingly, a *c* protein, *Puccinia* NPR1 interactor (PNPi) interacts with NPR1 to affect its association with TGA transcription factors. Through this action, downstream expression of *PR1* is significantly reduced. This putatively contributes to *P. striiformis* f. sp. *tritici* pathogenicity, leading to host susceptibility (Wang et al., 2016). In black poplar trees (*Populus nigra*), SA levels increased in plants infected with the biotrophic rust fungus, *Melampsora larici-populina*. Through the increased SA levels, the biosynthesis of the defence compound flavan-3-ol increased, which contributes to resistance against this rust pathogen (Ullah et al., 2019). Similarly, in Myrtaceae a recent study investigated the transcriptome of *E. grandis* infected with *A. psidii* at 1-dpi. The SA pathway was implicated in resistance against the pathogen, with this pathway highlighted in the resistant genotypes (Santos et al., 2020). Further research is needed to unravel the complex role that SA plays in resistance against *A. psidii* in eucalyptus.

1.6 Fungal pathogenicity and virulence mechanisms

Pathogens have evolved complex systems and mechanisms to overcome their host plant resistance, which includes secretion of molecules that suppress host defences (Hückelhoven, 2005). Virulence is defined as the degree of disease caused by a pathogenic organism, while pathogenicity is defined as the ability of a pathogenic organism to cause disease (Sacristan and García-Arenal, 2008). To further define virulence, Sacristan and García-Arenal (2008) states that it is the ability of a pathogen genotype to overcome a host resistance mechanism. Despite the significance of both virulence and pathogenicity, research has focused significantly on the ability of an organism to cause disease. Fungal pathogenicity genes are those that are described as being essential for plant disease development as alterations or deletions of these fungal genes results in reduced disease severity (van de Wouw and Howlett, 2011).

Fungal pathogenicity genes have been found in most stages of pathogenic infection, contributing plant disease at each stage of infection. Figure 1.4, adopted from Meng et al. (2009), shows a typical obligate biotrophic fungal infection cycle. A dispersed fungal spore attaches to the surface of the host plant, upon which host recognition takes place. A germ tube emerges from the fungal spore and elongates along the surface of the host plant. Following this, an appressorium forms from physical signals on the plant surface. A penetration peg emerges from the appressorium that can penetrate the surface of the host plant to gain access to the host cells. Subsequently, a mother haustoria is produced by the fungal pathogen. The haustoria is an infection structure that secretes fungal effectors and toxic compounds to

successfully suppress the host immune response, thereby reducing plant defence against pathogenic invasion. The haustoria is responsible for sequestering nutrients from the host cell for the growth and development of the fungus. Following successful host infection and hyphal growth, a lesion develops near the surface of the host plant where sporulation of newly developed spores can take place. These spores are then dispersed, and the infection cycle continues (Meng et al., 2009). Where appropriate, pathogenicity genes involved in key stages of the fungal infection process are discussed below.

1.6.1 Infection structures

The appressoria, specialised infection cells, are known to be involved in fungal pathogenicity. Rice blast fungus (*M. oryzae*) appressorium synthesis is associated with up-regulation of genes involved in host plant amino acid and protein degradation, suggesting these pathogenicity genes play crucial roles in causing disease in the host (Oh et al., 2008). In the hemi-biotrophic fungi *Colletotrichum graminicola,* researchers have identified key pathogenicity genes crucial to fungal development (Eisermann et al., 2019). Using rigorous approaches at identifying and characterising effector proteins of this pathogen, the authors found two genes in a cluster that are crucial to pathogen virulence. Deletion of these genes reduced efficiency of fungal appressorium penetration, due to inability to form penetration pegs and papillae, contributing to reduced virulence (Eisermann et al., 2019).

1.6.2 Host penetration

Cuticle and cell wall degrading enzymes (CWDE), among various other mechanisms, are ways in which pathogens degrade preformed barriers such as cutin and pectin in plants (Idnurm and Howlett, 2001). The degradation of cell walls facilitates the entry of pathogens into the host cells, followed by subsequent suppression of host defences and nutrient acquisition (Hückelhoven, 2005). CWDEs were shown to play significant roles in host colonisation of flax by flax rust (*M. lini*). The authors found the involvement of enzymes such as glucosidases, glucanases, pectin methyl esterases and mannosidase that facilitate degradation of cell walls to support pathogen colonisation and success in the host plant (Wu et al., 2019). In *E. grandis*, research has shown that the leaf cuticle wax layer undergoes enzymatic degradation following adhesion of *A. psidii* spores. *A. psidii* produces these toxic enzymes in the extracellular mucilaginous matrix in the spores (Xavier et al., 2015). To further support this, Quecine et al. (2016) found the involvement of *A. psidii* pathogenicity factors during the spore phase, putatively contributing to disease severity in susceptible guava (*P. guajava).* The ability of *A. psidii* to successfully breach the waxy layer of plant leaves by enzymatic degradation may contribute to host susceptibility seen among many different species.

1.6.3 Host colonisation and nutrient acquisition

Nutrient uptake is through the complex structure known as the haustoria. This structure is responsible for the secretion of compounds and proteins, including effector proteins, that function to suppress host immune responses, thereby facilitating the uptake of host-derived nutrients that support fungal growth and development (Voegele and Mendgen, 2003). Molecular studies on the role that haustoria play during the infection process and the genes expressed during the fungus-host interaction have previously been limited by the fact that haustoria can only be produced *in planta* (Voegele and Mendgen, 2003). With the development of genomic tools to study host-pathogen interactions at the molecular level, research has emerged on the unique roles these structures play in pathogenicity.

In *U. maydis*, the loss of a sucrose transporter, a protein that acquires host-derived sucrose, rendered the pathogen unable to cause disease (Wahl et al., 2010). This highlights the importance of acquisition of host-derived nutrients in pathogenicity. Haustoria specific gene expression in *Podosphaera xanthii* revealed up-regulation of genes associated with 1,3-β-D-glucan catabolism. The authors suggest these genes are regulated upon formation of the mother haustoria in the host plant or degradation of plant β-glucans, thereby promoting pathogenicity (Polonio et al., 2019). In *P. striiformis* f. sp. *tritici,* the haustoria plays crucial roles during the interaction with wheat. A recent study utilised RNA-seq to investigate the haustoria-derived transcriptome of this pathogen during the interaction with its host (Xu et al., 2020). The authors found up-regulation of genes associated with metabolic processes such as thiamine biosynthesis and glycolysis. Furthermore, host-induced gene silencing (HIGS) of various metabolism-related genes found reduced pathogenic growth and development within the host, putatively affecting pathogenicity. Haustoria-specific gene expression may also involve *in planta*-induced genes (*PIGs*). These *PIGs* primarily involve proteins that act as amino acid transporters, strengthening the importance of haustoria in nutrient acquisition and fungal pathogenicity (Voegele and Mendgen, 2003).

The haustoria play important roles in protecting the pathogen from ROS, thereby promoting pathogenicity (Polonio et al., 2019). In a study on the interaction between the obligate biotrophic rust fungus *Uromyces fabae-Vicia faba*, an increase in mannitol levels was observed. The authors suggest *U. fabae* utilises mannitol to scavenge ROS produced by the host immune response to mitigate the host defence response (Voegele et al., 2005). The role that *A. psidii* haustoria cells play during the interaction with its Myrtaceous hosts are yet to be elucidated. However, molecular studies with the newly released *A. psidii* genome (Tobias et al., 2021) will facilitate future studies to investigate the pathogenicity mechanisms employed by this structure through haustoria-specific expression studies.

1.6.4 Fungal effectors

Fungal effectors are proteins secreted by fungi, both pathogenic and mutualist, that can manipulate interactions with the host plant, typically reprogramming the host plant to allow successful fungal colonisation and proliferation, while contributing to suppressing host defence responses (Hogenhout et al., 2009; Mukhtar et al., 2011; Lo Presti et al., 2015). Fungal effectors can be toxic SMs that successfully suppress host plant defence responses or proteins that initiate cell death (Lo Presti et al., 2015). Through plant-pathogen protein-protein interactome networks, Mukhtar et al. (2011) concluded that virulence factors from two pathogens of different kingdoms interact with highly connected hubs in host plants. The authors provide evidence to suggest that plants possess overlapping cellular machinery that act as targets for effectors from pathogens with drastically different lifestyles and that plants do not possess pathogen-lifestyle-specific targets (Mukhtar et al., 2011).

There are different widely accepted hypotheses suggesting how a host plant interacts with fungal effectors. These small, secreted proteins can have a gene-for-gene relationship with a host target protein, in which case they are termed avirulence (Avr) factors. In the gene-for-gene theory, a host R protein recognises a pathogen Avr protein, and an immune response is induced. When the pathogen Avr protein is altered and no longer recognised by the plant R protein, the plant does not detect pathogenic invasion and subsequent disease occurs (Van Der Biezen and Jones, 1998; van de Wouw and Howlett, 2011). Alternatively, R proteins can be activated by recognition of the effects of the virulence or effector proteins. This is indirectly through the changes these pathogen proteins make to the host targets. The latter is known as the ‘Guard Hypothesis’ (Van Der Biezen and Jones, 1998). Mukhtar et al. (2011) provides evidence for the Guard Hypothesis, in which they identified an indirect association between pathogen effectors and host R proteins.

Similar to the Guard Hypothesis, is the Decoy Model (van der Hoorn and Kamoun, 2008). Host proteins can either act as target or helper proteins. Target proteins are those that are directly targeted by effector proteins to elicit changes in host immunity responses. Helper proteins are those that facilitate *in planta* function of the effector protein and in its absence, the pathogen exhibits reduced pathogenicity. A recent paper studied the interaction between an obligate biotrophic rust pathogen *M. larici-populina* and poplar to determine the role of an identified effector protein, Mlp124357 (Madina et al., 2020). Madina et al. (2020) found transgenic *Arabidopsis* expressing this effector gene lead to increased susceptibility to bacterial and oomycete infection. Furthermore, the effector protein interacted with a host protein, *Arabidopsis* protein disulfide isomerase-11 (AtPDI-11), to localise to the tonoplast of the host cells to facilitate infection and increase plant susceptibility (Madina et al.,

2020). The authors suggest this host protein acts as a helper to facilitate *in planta* function of the effector protein.

Another study looked at an effector protein of *M. larici-populina,* Mlp37347 (Rahman et al., 2021). The authors found this protein, secreted by this leaf rust pathogen during infection of poplar, manipulates the plasmodesmata of host cells to facilitate the fungal infection capabilities of cells neighbouring the initial infection site. Cells harbouring this effector protein at the plasmodesmata presented with reduced callose deposition and plants showed increased susceptibility to a biotrophic pathogen, *Hyalonoperospora arabidopsidis.* While pathogens manipulating the plasmodesmata have been previously reported, this is the first study to show this interaction in rust fungi. The authors suggest that the pathogen manipulates glucan catabolism, thereby facilitating the reduction in callose deposition at the plasmodesmata, which causes increased susceptibility to the host plants (Rahman et al., 2021).

Despite the significance of pathogen effectors and the profound effect these proteins have on plant immunity, we know comparatively little about eukaryotic effectors and even less about effectors of rust fungi. Significant research is needed to elucidate the mechanisms these proteins utilise to initiate disease in their target hosts. Identifying candidate effector proteins and determining the *in planta* role in pathogenicity will contribute to research on the development of novel, targeted control strategies to mitigate disease. Various online databases documenting pathogenicity and virulence genes are publicly available. For example, the PHI (pathogen-host interaction, http://www.phi-base.org/) database is a powerful tool that documents functionally characterised pathogenicity and virulence genes of specific fungal species (Urban et al., 2015).

1.7 Myrtaceae molecular responses to *Austropuccinia psidii*

The advent of omics tools will facilitate studies in these non-model organisms and are invaluable to unravelling the complex interactions between *A. psidii* and its hosts. These tools include genomics, transcriptomics, proteomics, metabolomics and effectoromics. As a result, significant efforts have been made in functional genetics and gene expression studies to identify biological pathways and genes significantly contributing to resistance against *A. psidii* (Junghans et al., 2003; Moon et al., 2007; Mamani et al., 2010; Alves et al., 2012; Thumma et al., 2013; Laia et al., 2015; Butler et al., 2016; Hsieh et al., 2018; Tobias et al., 2018; Soewarto et al., 2019b; Santos et al., 2020). With increasing knowledge on the molecular dialogue that exists between the host and pathogen, we can begin to develop robust control strategies to mitigate the effect of myrtle rust. This includes informed selective

breeding for resistant traits (e.g., resistant quantitative trait loci, QTLs), biological pathways involved in defence and pyramiding of resistance genes (*R*-genes). The *Austropuccinia psidii* reference genome (Tobias et al., 2021) will further our knowledge on the dialogue myrtle rust contributes to the crosstalk with its host plants, with this providing valuable insights into novel pathogen targets for control. These molecular studies are of vital importance, as host resistance remains the most cost-effective and long-term control strategy against *A. psidii* (Glen et al., 2007). The following section discusses our current knowledge on the host-pathogen molecular crosstalk, and includes genomic, transcriptomic, and proteomic and metabolomic studies.

In *E. grandis,* a major locus associated with resistance to *A. psidii* was identified on linkage group 3 (Junghans et al., 2003; Mamani et al., 2010), with this locus, termed *Puccinia psidii resistance 1* (*Ppr1,* as per the previous classification of *A. psidii*), harbouring numerous disease resistance genes that may contribute to the phenotypes observed (Thumma et al., 2013). This locus has been used in molecular breeding programmes to engineer rust resistance in *E. grandis*. Unfortunately, genotypes harbouring *Ppr1* were found to be susceptible to certain *A. psidii* strains, suggesting *Ppr1* was not efficient enough to provide resistance to this pathogen (Graça et al., 2011). With genetic mapping, Alves et al. (2012) identified additional QTLs that played additive and non-additive roles in resistance in *Eucalyptus* to *A. psidii*. These minor loci were able to explain approximately 29.8% and 44.8% of the phenotypic variation observed between different hybrids of *Eucalyptus.* Additionally, four loci identified in *E. globulus* were found to be associated with *A. psidii* resistance, with these loci determining whether the plant exhibits symptoms (*Ppr 2* and *Ppr3*) and displays an HR (*Ppr4* and *Ppr5*) (Butler et al., 2016). Furthermore, Butler et al. (2019) identified novel, independent QTLs associated with *A. psidii* resistance in *Corymbia*. The identification of various QTLs associated with disease resistance in different species highlights the complex nature of disease resistance against *A. psidii*. These QTLs suggest that *A. psidii* resistance is not controlled by a single gene but various genes underlying many QTLs contributing to the phenotypes observed. Future research needs to involve studies on the expression of genes underlying these loci, to determine important genes involved in the interaction and how these contribute to the resistance observed.

Yong et al. (2021) performed a genome wide association study (GWAS) to investigate *E. oliqua* resistance against myrtle rust. The authors found SNP markers associated with different resistances against *A. psidii*, including disease severity, symptoms of infection, HR, and pustule formation. These SNP markers were located near resistance loci *Ppr1-5.* Numerous genes were identified throughout the study, such as BR signalling kinase 3 associated with the presence or absence of a symptomatic

response, genes associated with the presence or absence of an HR and beta-galactosidase genes associated with the severity of disease (Yong et al., 2021).

There are many papers that have emerged over recent years that study the transcriptome of Myrtaceous species upon interactions with *A. psidii* (Hsieh et al., 2018; Tobias et al., 2018; Santos et al., 2020; Swanepoel et al., 2021). These papers have corroborated the complex nature of the interactions between the host and *A. psidii*. Tobias et al. (2018) studied the transcriptome of *S. luehmannii* upon infection with *A. psidii* at 1- and 2-dpi, in four resistant and susceptible individuals. They found that susceptible *S. luehmannii* were unable to detect *A. psidii* invasion early enough to elicit an effective immune response against the pathogen. Early recognition of invading microbes is crucial to a rapid immune response, as seen in resistant *S. luehmannii*. Furthermore, two putative toll/interleukin-1-NBS-LRR-type (TIR-NBS-LRR-type) resistance genes were up-regulated in resistant plants, with similarities likened to Eucgr.C01654 and Eucgr.C01968 in *E. grandis* on linkage group 3, within the confidence interval of *Ppr1*. This suggests an involvement of these *R*-genes in resistance against *A. psidii*.

Finally, the involvement of secondary metabolic pathways was associated with *A. psidii* resistance, with up-regulation of genes associated in the phenylpropanoid pathway (Tobias et al., 2018). Similar responses were observed when studying the interactions between *Melaleuca quinquenervia* upon infection with *A. psidii* (Hsieh et al., 2018). The authors found significant involvement of the phenylpropanoid pathway. Pathogen-derived transcripts associated with haustoria development were identified in susceptible plants at 5-dpi, suggesting significant pathogen proliferation had occurred in susceptible hosts, with these responses absent in resistant hosts. Susceptible plants were unable to initiate rapid defence responses against *A. psidii* invasion, as observed in *S. luehmannii*, and significant defence-related expression was only observed at 5-dpi, which may be too late to prevent the spread of infection. In resistant individuals, *PR* expression was significantly higher, with these genes putatively contributing to disease resistance (Hsieh et al., 2018).

In *E. grandis*, constitutive expression of defence-related genes is hypothesised to contribute to disease resistance (Santos et al., 2020). The background expression of resistant and susceptible *E. grandis* genotypes was significantly different without inoculation with *A. psidii*, suggesting the importance of constitutive expression. Constitutive expression in resistant plants includes genes relating to SA-mediated responses, photosynthesis, and protein kinase leucine-rich receptors. Constitutive overexpression of *R*-genes was associated with the resistant genotype. The authors hypothesise these responses contribute to basal immunity against *A. psidii*. As seen in *M. quinquenervia* and *S.*

luehmannii, susceptible *E. grandis* is not efficient at rapid responses following *A. psidii* infection, and susceptible genotypes had reduced expression of defence-related genes in response to *A. psidii* infection. These are the first studies to investigate the changes in the transcriptome of Myrtaceous species upon infection with *A. psidii* and provide insights into mechanisms putatively contributing to disease resistance.

A recent paper investigated the interactions between *A. psidii* and *E. grandis* by combining proteomics and metabolomics to determine early responses to infection (Sekiya et al., 2021). Resistance in *E. grandis* was associated with enzymes and metabolites involved in the phenylpropanoid pathway, corroborating findings of previous studies that suggested the importance of this pathway in disease resistance. An absence of these responses in susceptible *E. grandis* genotypes led to the progression of disease symptoms. Through functional enrichment, at 12-hpi in resistant genotypes, identified mechanisms included the involvement of the photosynthetic pathway and oxidative burst responses. While susceptible genotypes showed similar responses, early accumulation of these metabolites and ineffective downstream control led to susceptible plants succumbing to *A. psidii* infection (Sekiya et al., 2021).

There is a wealth of information emerging on the host dialogue with myrtle rust, with comparatively few studies investigating the pathogen molecular responses. Quecine et al. (2016) investigated the proteome of resistant *E. grandis* and susceptible *Psidium guajava* (guava). The authors isolated various proteins from the spores of *A. psidii* collected from these species. In the susceptible guava samples, proteins associated with fungal virulence were found, which included malate dehydrogenase enzymes, enolases, heat shock proteins, peptidases and proteases. Proteins isolated from spores on resistance *E. grandis* included translation proteins and proteins involved in folding. This suggests that there is a difference in the proteomes of myrtle rust infecting different hosts and this provides insights into how the pathogen elicits disease on susceptible hosts (Quecine et al., 2016).

These studies highlight the complex nature of *A. psidii*-host interactions, with various significant defence pathways found throughout genomic, transcriptomic, metabolomic and proteomic analyses. It is important to unravel the complex crosstalk that exists between host and pathogen, as breeding plants and engineering hybrids for rust resistance is paramount in mitigating the effects of this pathogen. Rust resistance relies on a multifaceted, integrated system and further research is needed to elucidate these mechanisms.

1.8 Conclusion and future perspectives

Rust fungi are obligate biotrophs, which complicates genetic molecular studies as they require a live host to grow and replicate without cultivation on artificial media. As a result, studies on the molecular nature of biotrophic fungi are few and less advanced than that of other pathogenic organisms, such as necrotrophs. In recent years, the explosion of the genomics era has facilitated studies on biotrophic fungi (Bakkeren and Szabo, 2020), with the complexity of these systems and their molecular dialogues with their hosts explored on new levels. The overarching goal is to find candidate genes putatively involved in host infection for functional studies. This will reveal the role gene products play in successful host colonisation and pathogenicity. Due to the large and complex nature of rust fungi, with gene numbers as high as 28,000 (Aime et al., 2017), integrating multiple levels of studies to identify candidate genes is essential. By narrowing large lists of genes to a smaller subset involved in an interaction, functional studies to characterise these genes will be more targeted and successful. With this knowledge, disease control strategies can be more effective at managing these destructive pathogens.

The *A. psidii*-host interactions are complex systems, with the interactions differing between different species and in some cases, within the same species. The ability of *A. psidii* to cause such varying degrees of disease on a wide range of different host species is an important avenue to investigate, as it may lead to the development of novel and targeted control strategies. Pathogenicity factors facilitating the ability of myrtle rust to infect a wide range of species can be targeted, while virulence factors determining the degree of infection may lead to the engineering, selecting and breeding for host resistance. Further research is required to identify and characterise these genes, which will be significantly facilitated following the release of the *Austropuccinia psidii* genome (Tobias et al., 2021). Due to the imminent threat that this pathogen poses to the South African forestry industry (Roux et al., 2015), future studies are required to investigate the mechanisms underlying host resistance and susceptibility in commonly planted seed lots as this will guide selective breeding for resistance.

1.9 References

Adendorff, R., and Rijkenberg, F.H.J. (2000). Scanning electron microscopy of direct host leaf penetration by urediospore-derived infection structures of *Phakopsora apoda*. *Mycological Research* 104**,** 317-324. doi: 10.1017/S0953756299001203.

Aggarwal, K.K., Khanuja, S.P.S., Ahmad, A., Santha Kumar, T.R., Gupta, V.K., and Kumar, S. (2002). Antimicrobial activity profiles of the two enantiomers of limonene and carvone isolated from the oils of *Mentha spicata* and *Anethum sowa*. *Flavour and Fragrance Journal* 17**,** 59-63. doi: 10.1002/ffj.1040.

Aime, M.C., Mctaggart, A.R., Mondo, S.J., and Duplessis, S. (2017). "Phylogenetics and phylogenomics of rust fungi," in *Advances in Genetics,* eds. J.P. Townsend & Z. Wang. (Academic Press: Elsevier), 267-307.

Alfenas, A.C., Maffia, L.A., Macabeu, A.J., and Sartorio, R.C. (1993). Efficiency of triadimenol, oxycarboxin and diniconazole for rust (*Puccinia psidii*) control in coppice of *Eucalyptus cloeziana* under field conditions. *Revista Arvore* 17**,** 247-263.

Ali, S., Ganai, B.A., Kamili, A.N., Bhat, A.A., Mir, Z.A., Bhat, J.A., et al. (2018). Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiological Research* 212-213**,** 29-37. doi: 10.1016/j.micres.2018.04.008.

Alves, A.A., Rosado, C.C.G., Faria, D.A., Da Silva Guimarães, L.M., Lau, D., Brommonschenkel, S.H., et al. (2012). Genetic mapping provides evidence for the role of additive and non-additive QTLs in the response of inter-specific hybrids of *Eucalyptus* to *Puccinia psidii* rust infection. *Euphytica* 183**,** 27-38. doi: 10.1007/s10681-011-0455-5.

Amorim, E.P.R., Pio-Ribeiro, G., Menezes, M., and Coelho, R.S.B. (1993). The pathogenicity and hyperparasitic action of *Fusarium decemcellulare* on *Puccinia psidii* in guava (*Psidium guajava*). *Fitopatologia Brasileira* 18**,** 226-229.

Antico, C.J., Colon, C., Banks, T., and Ramonell, K.M. (2012). Insights into the role of jasmonic acid-mediated defenses against necrotrophic and biotrophic fungal pathogens. *Frontiers in Biology* 7**,** 48-56. doi: 10.1007/s11515-011-1171-1.

Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nature Immunology* 6**,** 973-979. doi: 10.1038/ni1253.

Azaiez, A., Boyle, B., Levée, V., and Séguin, A. (2009). Transcriptome profiling in hybrid poplar following interactions with *Melampsora* rust fungi. *Molecular Plant-Microbe Interactions* 22**,** 190-200. doi: 10.1094/mpmi-22-2-0190.

Bakkeren, G., and Szabo, L.J. (2020). Progress on molecular genetics and manipulation of rust fungi. *Phytopathology* 110**,** 532-543. doi: 10.1094/PHYTO-07-19-0228-IA.

Bari, R., and Jones, J.D.G. (2009). Role of plant hormones in plant defence responses. *Plant Molecular Biology* 69**,** 473-488. doi: 10.1007/s11103-008-9435-0.

Beenken, L. (2017). *Austropuccinia*: a new genus name for the myrtle rust *Puccinia psidii* placed within the redefined family Sphaerophragmiaceae (Pucciniales). *Phytotaxa* 297**,** 53-61. doi: 10.11646/phytotaxa.297.1.5.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* 60**,** 379-406. doi: 10.1146/annurev.arplant.57.032905.105346.

Bonora, F.S., Nahrung, H.F., Hayes, R.A., Scharaschkin, T., Pegg, G.S., and Lee, D.J. (2020). Changes in leaf chemistry and anatomy of *Corymbia citriodora subsp. variegata* (Myrtaceae) in response to native and exotic pathogens. *Australasian Plant Pathology* 49**,** 641-653. doi: 10.1007/s13313-020-00739-z.

Booth, T.H., Old, K.M., and Jovanovic, T. (2000). A preliminary assessment of high risk areas for *Puccinia psidii* (*Eucalyptus* rust) in the Neotropics and Australia. *Agriculture, Ecosystems & Environment* 82**,** 295-301. doi: 10.1016/S0167-8809(00)00233-4.

Butler, J.B., Freeman, J.S., Vaillancourt, R.E., Potts, B.M., Glen, M., Lee, D.J., et al. (2016). Evidence for different QTL underlying the immune and hypersensitive responses of *Eucalyptus globulus* to the rust pathogen *Puccinia psidii*. *Tree Genetics & Genomes* 12**,** 39. doi: 10.1007/s11295-016-0987-x.

Butler, J.B., Potts, B.M., Vaillancourt, R.E., Lee, D.J., Pegg, G.S., and Freeman, J.S. (2019). Independent QTL underlie resistance to the native pathogen *Quambalaria pitereka* and the exotic pathogen *Austropuccinia psidii* in *Corymbia*. *Tree Genetics & Genomes* 15**,** 72. doi: 10.1007/s11295-019-1378-x.

Carnegie, A., and Cooper, K. (2011). Emergency response to the incursion of an exotic myrtaceous rust in Australia. *Australasian Plant Pathology* 40**,** 346-359. doi: 10.1007/s13313-011-0066-6.

Carnegie, A.J., Lidbetter, J.R., Walker, J., Horwood, M.A., Tesoriero, L., Glen, M., et al. (2010). *Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia. *Australasian Plant Pathology* 39**,** 463-466. doi: 10.1071/AP10102.

Chandrashekara, K., Manivannan, S., Chandrashekara, C., and Chakravarthi, M. (2012). "Biological control of plant disease," in *Eco-friendly innovative approaches in plant disease management,* eds. V.K. Singh, Y. Singh & A. Singh. (New Delhi, India: International Book Distributors), 147-166.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J., Lorenzo, O., et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448**,** 666-671. doi: 10.1038/nature06006.

Christie, N., Tobias, P.A., Naidoo, S., and KüLheim, C. (2015). The *Eucalyptus grandis* NBS-LRR gene family: physical clustering and expression hotspots. *Frontiers in Plant Science* 6**,** 1238. doi: 10.3389/fpls.2015.01238.

Coutinho, T.A., Wingfield, M.J., Alfenas, A.C., and Crous, P.W. (1998). *Eucalyptus* Rust: A disease with the potential for serious international implications. *Plant Disease* 82**,** 819-825. doi: 10.1094/PDIS.1998.82.7.819.

Couto, D., and Zipfel, C. (2016). Regulation of pattern recognition receptor signalling in plants. *Nature Reviews Immunology* 16**,** 537-552. doi: 10.1038/nri.2016.77.

De Bruyne, L., Höfte, M., and De Vleesschauwer, D. (2014). Connecting growth and defense: The emerging roles of brassinosteroids and gibberellins in plant innate immunity. *Molecular Plant* 7**,** 943-959. doi: 10.1093/mp/ssu050.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueler, M.J., et al. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions* 18**,** 923-937. doi: 10.1094/MPMI-18-0923.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics* 11**,** 539-548. doi: 10.1038/nrg2812.

dos Santos, I.B., Lopes, M.D.S., Bini, A.P., Tschoeke, B.a.P., Verssani, B.a.W., Figueredo, E.F., et al. (2019). The *Eucalyptus* cuticular waxes contribute in preformed defense against *Austropuccinia psidii*. *Frontiers in Plant Science* 9**,** 1978. doi: 10.3389/fpls.2018.01978.

Du Plessis, E., Granados, G.M., Barnes, I., Ho, W.H., Alexander, B.J.R., Roux, J., et al. (2019). The pandemic strain of *Austropuccinia psidii* causes myrtle rust in New Zealand and Singapore. *Australasian Plant Pathology* 48**,** 253-256. doi: 10.1007/s13313-019-0624-x.

Du Plessis, E., Mctaggart, A.R., Granados, G.M., Wingfield, M.J., Roux, J., Ali, M.I.M., et al. (2017). First report of myrtle rust caused by *Austropuccinia psidii* on *Rhodomyrtus tomentosa* (Myrtaceae) from Singapore. *Plant Disease* 101**,** 1676. doi: 10.1094/PDIS-04-17-0530-PDN.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology* 42**,** 185-209. doi: 10.1146/annurev.phyto.42.040803.140421.

Eisermann, I., Weihmann, F., Krijger, J.J., Kroling, C., Hause, G., Menzel, M., et al. (2019). Two genes in a pathogenicity gene cluster encoding secreted proteins are required for appressorial penetration and infection of the maize anthracnose fungus *Colletotrichum graminicola*. *Environ. Microbiol.* 21**,** 4773-4791. doi: 10.1111/1462-2920.14819.

Fensham, R.J., Carnegie, A.J., Laffineur, B., Makinson, R.O., Pegg, G.S., and Wills, J. (2020). Imminent extinction of Australian Myrtaceae by fungal disease. *Trends in Ecology & Evolution* 35**,** 7. doi: 10.1016/j.tree.2020.03.012.

Garnas, J.R., Hurley, B.P., Slippers, B., and Wingfield, M.J. (2012). Biological control of forest plantation pests in an interconnected world requires greater international focus. *International Journal of Pest Management* 58**,** 211-223. doi: 10.1080/09670874.2012.698764.

Giblin, F. (2013). Myrtle rust report: New Caledonia. *University of the Sunshine Coast, Maroochydore, Queensland*.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* 43**,** 205-227. doi: 10.1146/annurev.phyto.43.040204.135923.

Glen, M., Alfenas, A.C., Zauza, E.a.V., Wingfield, M.J., and Mohammed, C. (2007). *Puccinia psidii*: A threat to the Australian environment and economy - A review. *Australasian Plant Pathology* 36**,** 1-16. doi: 10.1071/AP06088.

Graça, R.N., Aun, C.P., Guimarães, L.M.S., Rodrigues, B.V.A., Zauza, E.a.V., and Alfenas, A.C. (2011). A new race of *Puccinia psidii* defeats rust resistance in eucalypt. *Australasian Plant Pathology* 40**,** 442-447. doi: 10.1007/s13313-011-0056-8.

Grattapaglia, D. (2008). "Genomics of *Eucalyptus*, a global tree for energy, paper, and wood," in *Genomics of Tropical Crop Plants*. Springer), 259-298.

Grattapaglia, D., Vaillancourt, R.E., Shepherd, M., Thumma, B.R., Foley, W., Külheim, C., et al. (2012). Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. *Tree Genetics & Genomes* 8**,** 463-508. doi: 10.1007/s11295-012-0491-x.

Grove, M.D., Spencer, G.F., Rohwedder, W.K., Mandava, N., Worley, J.F., Warthen, J.D., et al. (1979). Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature* 281**,** 216-217. doi: 10.1038/281216a0.

He, J.-X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.-Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 99**,** 10185-10190. doi: 10.1073/pnas.152342599.

He, Y., Zhang, H., Sun, Z., Li, J., Hong, G., Zhu, Q., et al. (2017). Jasmonic acid-mediated defense suppresses brassinosteroid-mediated susceptibility to rice black streaked dwarf virus infection in rice. *New Phytologist* 214**,** 388-399. doi: 10.1111/nph.14376.

Hill, K.D., and Johnson, L.A. (1995). Systematic studies in the eucalypts 7. A revision of the bloodwoods, genus *Corymbia* (Myrtaceae). *Telopea* 6**,** 185-504. doi: 10.7751/telopea19953017.

Hogenhout, S.A., Van Der Hoorn, R.a.L., Terauchi, R., and Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interactions* 22**,** 115-122. doi: 10.1094/mpmi-22-2-0115.

Hsieh, J.-F., Chuah, A., Patel, H.R., Sandhu, K.S., Foley, W.J., and KüLheim, C. (2018). Transcriptome profiling of *Melaleuca quinquenervia* challenged by myrtle rust reveals differences in defense responses among resistant individuals. *Phytopathology* 108**,** 495-509. doi: 10.1094/PHYTO-09-17-0307-R.

Hsieh, J.-F., Krause, S.T., Kainer, D., Degenhardt, J., Foley, W.J., and Külheim, C. (2021). Characterization of terpene biosynthesis in *Melaleuca quinquenervia* and ecological consequences of terpene accumulation during myrtle rust infection. *Plant-Environment Interactions* 2**,** 177-193. doi: 10.1002/pei3.10056.

Hückelhoven, R. (2005). Powdery mildew susceptibility and biotrophic infection strategies. *FEMS Microbiology Letters* 245**,** 9-17. doi: 10.1016/j.femsle.2005.03.001.

Hyde, K.D., Al-Hatmi, A.M.S., Andersen, B., Boekhout, T., Buzina, W., Dawson, T.L., Jr., et al. (2018). The world's ten most feared fungi. *Fungal Diversity* 93**,** 161-194. doi: 10.1007/s13225-018-0413-9.

Idnurm, A., and Howlett, B.J. (2001). Pathogenicity genes of phytopathogenic fungi. *Molecular Plant Pathology* 2**,** 241-255. doi: 10.1046/j.1464-6722.2001.00070.x.

Iglesias-Trabado, G., and Wilstermann, D. (2008). *Eucalyptus universalis, global cultivated eucalypt forests map 2008, Version 1.0.1.* GIT Forestry Consulting's Eucalyptologics.

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biological Research* 52**,** 39. doi: 10.1186/s40659-019-0246-3.

Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. *Nature* 444**,** 323-329. doi: 10.1038/nature05286.

Junghans, D.T., Alfenas, A.C., Brommonschenkel, S.H., Oda, S., Mello, E.J., and Grattapaglia, D. (2003). Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. *Theoretical and Applied Genetics* 108**,** 175-180. doi: 10.1007/s00122-003-1415-9.

Kawanishi, T., Uematsu, S., Kakishima, M., Kagiwada, S., Hamamoto, H., Horie, H., et al. (2009). First report of rust disease on ohia and the causal fungus, *Puccinia psidii*, in Japan. *Journal of General Plant Pathology* 75**,** 428-431. doi: 10.1007/s10327-009-0202-0.

Keszei, A., Brubaker, C.L., and Foley, W.J. (2008). A molecular perspective on terpene variation in Australian Myrtaceae. *Australian Journal of Botany* 56**,** 197-213. doi: 10.1071/BT07146.

Khare, S., Singh, N., Singh, A., Hussain, I., Niharika, K., Yadav, V., et al. (2020). Plant secondary metabolites synthesis and their regulations under biotic and abiotic constraints. *Journal of Plant Biology* 63**,** 203-216. doi: 10.1007/s12374-020-09245-7.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., et al. (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. *The Plant Journal* 26**,** 509-522. doi: 10.1046/j.1365-313x.2001.01050.x.

Kovalchuk, A., Keriö, S., Oghenekaro, A.O., Jaber, E., Raffaello, T., and Asiegbu, F.O. (2013). Antimicrobial defenses and resistance in forest trees: challenges and perspectives in a genomic era. *Annual Review of Phytopathology* 51**,** 221-244. doi: 10.1146/annurev-phyto-082712-102307.

Ladiges, P.Y. (1997). "Phylogenetic history and classification of eucalypts," in *Eucalypt Ecology: individuals to ecosystems*. (Cambridge, UK: Cambridge University Press), 16-29.

Lai, Y., and Eulgem, T. (2018). Transcript-level expression control of plant NLR genes. *Molecular Plant Pathology* 19**,** 1267-1281. doi: 10.1111/mpp.12607.

Laia, M.L., Alfenas, A.C., Brommonschenkel, S.H., Oda, S., De Melo, E.J., De Araújo Silva, I.M., et al. (2015). Cloning and characterization of gene-resistant analogs (RGAs) involved in rust (*Puccinia psidii*)

resistance in *Eucalyptus grandis*. *Journal of Forestry Research* 26**,** 711-718. doi: 10.1007/s11676-015-0049-5.

Lira, V.L., Da Silva Santos, A.C., Tiago, P.V., De Oliveira, N.T., and De Moura, R.M. (2019). Hyperparasitism of *Fusarium* spp. in *Austropuccinia psidii* in "Jambo-do-Para". *Summa Phytopathologica* 45**,** 204-206. doi: 10.1590/0100-5405/187593.

Liu, J.J., Williams, H., Li, X.R., Schoettle, A.W., Sniezko, R.A., Murray, M., et al. (2017). Profiling methyl jasmonate-responsive transcriptome for understanding induced systemic resistance in whitebark pine (*Pinus albicaulis*). *Plant Molecular Biology* 95**,** 359-374. doi: 10.1007/s11103-017-0655-z.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., et al. (2015). Fungal effectors and plant susceptibility. *Annual Review of Plant Biology* 66**,** 513-545. doi: 10.1146/annurev-arplant-043014-114623.

Loope, L. (2010). "A summary of information on the rust *Puccinia psidii* Winter (guava rust) with emphasis on means to prevent introduction of additional strains to Hawaii". US Geological Survey).

Low, P.S., and Merida, J.R. (1996). The oxidative burst in plant defense: function and signal transduction. *Physiologia Plantarum* 96**,** 533-542. doi: 10.1111/j.1399-3054.1996.tb00469.x.

Madina, M.H., Rahman, M.S., Huang, X., Zhang, Y., Zheng, H., and Germain, H. (2020). A poplar rust effector protein associates with protein disulfide isomerase and enhances plant susceptibility. *Biology* 9**,** 294. doi: 10.3390/biology9090294.

Mamani, E.M., Bueno, N.W., Faria, D.A., Guimarães, L.M., Lau, D., Alfenas, A.C., et al. (2010). Positioning of the major locus for *Puccinia psidii* rust resistance (*Ppr1*) on the *Eucalyptus* reference map and its validation across unrelated pedigrees. *Tree Genetics & Genomes* 6**,** 953-962. doi: 10.1007/s11295-010-0304-z.

Mangwanda, R., Myburg, A.A., and Naidoo, S. (2015). Transcriptome and hormone profiling reveals *Eucalyptus grandis* defence responses against *Chrysoporthe austroafricana*. *BMC Genomics* 16**,** 319. doi: 10.1186/s12864-015-1529-x.

Marlatt, R.B., and Kimbrough, J.W. (1979). *Puccinia psidii* on *Pimenta dioica* in south Florida. *Plant Disease Reporter* 63**,** 510-512.

Mctaggart, A.R., Du Plessis, E., Roux, J., Barnes, I., Fraser, S., Granados, G.M., et al. (2020). Sexual reproduction in populations of *Austropuccinia psidii*. *European Journal of Plant Pathology* 156**,** 537-545. doi: 10.1007/s10658-019-01903-y.

Mctaggart, A.R., Roux, J., Granados, G.M., Gafur, A., Tarrigan, M., Santhakumar, P., et al. (2016a). Rust (*Puccinia psidii*) recorded in Indonesia poses a threat to forests and forestry in South-East Asia. *Australasian Plant Pathology* 45**,** 83-89. doi: 10.1007/s13313-015-0386-z.

Mctaggart, A.R., Shivas, R.G., Van Der Nest, M.A., Roux, J., Wingfield, B.D., and Wingfield, M.J. (2016b). Host jumps shaped the diversity of extant rust fungi (Pucciniales). *New Phytologist* 209**,** 1149-1158. doi: 10.1111/nph.13686.

Mctaggart, A.R., Shuey, L.S., Granados, G.M., Du Plessis, E., Fraser, S., Barnes, I., et al. (2018). Evidence that *Austropuccinia psidii* may complete its sexual life cycle on Myrtaceae. *Plant Pathology* 67**,** 729-734. doi: 10.1111/ppa.12763.

Meng, S., Torto-Alalibo, T., Chibucos, M.C., Tyler, B.M., and Dean, R.A. (2009). Common processes in pathogenesis by fungal and oomycete plant pathogens, described with Gene Ontology terms. *BMC Microbiology* 9**,** S7. doi: 10.1186/1471-2180-9-S1-S7.

Messal, M., Slippers, B., Naidoo, S., Bezuidt, O., and Kemler, M. (2019). Active fungal communities in asymptomatic *Eucalyptus grandis s*tems differ between a susceptible and resistant clone. *Microorganisms* 7**,** 16. doi: 10.3390/microorganisms7100375.

Meyer, F.E., Shuey, L.S., Naidoo, S., Mamni, T., Berger, D.K., Myburg, A.A., et al. (2016). Dual RNA-sequencing of *Eucalyptus nitens* during *Phytophthora cinnamomi* challenge reveals pathogen and host factors influencing compatibility. *Frontiers in Plant Science* 7**,** 191. doi: 10.3389/fpls.2016.00191.

Minchinton, E.J., Smith, D., Hamley, K., and Donald, C. (2014). Myrtle rust in Australia. *Acta Horticulturae***,** 89-90. doi: 10.17660/ActaHortic.2014.1055.19.

Mishina, T.E., and Zeier, J.R. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *The Plant Journal* 50**,** 500-513. doi: 10.1111/j.1365-313X.2007.03067.x.

Mishra, J., Srivastava, R., Trivedi, P.K., and Verma, P.C. (2020). Effect of virus infection on the secondary metabolite production and phytohormone biosynthesis in plants. *3 Biotech* 10**,** 547. doi: 10.1007/s13205-020-02541-6.

Moffat, C.S., Ingle, R.A., Wathugala, D.L., Saunders, N.J., Knight, H., and Knight, M.R. (2012). ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against *Botrytis cinerea* in Arabidopsis. *PloS One* 7**,** e35995. doi: 10.1371/journal.pone.0035995.

Montreal Process Implementation Group for Australia and National Forest Inventory Steering Committee (2018). "Australia's state of the forests report 2018". (Canberra, December. CC BY 4.0: ABARES).

Moon, D.H., Salvatierra, G.R., Caldas, D.G.G., Gallo De Carvalho, M.C.C., Carneiro, R.T., Franceschini, L.V.M., et al. (2007). Comparison of the expression profiles of susceptible and resistant *Eucalyptus grandis* exposed to *Puccinia psidii* Winter using SAGE. *Functional Plant Biology* 34**,** 1010. doi: 10.1071/FP07094.

Morin, L., Talbot, M.J., and Glen, M. (2014). Quest to elucidate the life cycle of *Puccinia psidii* sensu lato. *Fungal Biology* 118**,** 253-263. doi: 10.1016/j.funbio.2013.12.004.

Mortazavi, A., Williams, B.A., Mccue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods* 5**,** 621. doi: 10.1038/nmeth.1226.

Mukhtar, M.S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333**,** 596-601. doi: 10.1126/science.1203659.

Myburg, A.A., Grattapaglia, D., Tuskan, G.A., Hellsten, U., Hayes, R.D., Grimwood, J., et al. (2014). The genome of *Eucalyptus grandis*. *Nature* 510**,** 356. doi: 10.1038/nature13308.

Naidoo, R., Ferreira, L., Berger, D.K., Myburg, A.A., and Naidoo, S. (2013). The identification and differential expression of *Eucalyptus grandis* pathogenesis-related genes in response to salicylic acid and methyl jasmonate. *Frontiers in Plant Science* 4**,** 43. doi: 10.3389/fpls.2013.00043.

Naidoo, S., KüLheim, C., Zwart, L., Mangwanda, R., Oates, C.N., Visser, E.A., et al. (2014). Uncovering the defence responses of *Eucalyptus* to pests and pathogens in the genomics age. *Tree Physiology* 34**,** 931-943. doi: 10.1093/treephys/tpu075.

Naidoo, S., Slippers, B., Plett, J.M., Coles, D., and Oates, C.N. (2019). The road to resistance in forest trees. *Frontiers in Plant Science* 10**,** 273. doi: 10.3389/fpls.2019.00273.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., et al. (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *The Plant Journal* 33**,** 887-898. doi: 10.1046/j.1365-313x.2003.01675.x.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* 198**,** 249-266. doi: 10.1111/j.0105-2896.2004.0119.x.

Oates, C.N., Külheim, C., Myburg, A.A., Slippers, B., and Naidoo, S. (2015). The transcriptome and terpene profile of *Eucalyptus grandis* reveals mechanisms of defense against the insect pest, *Leptocybe invasa*. *Plant and Cell Physiology* 56**,** 1418-1428. doi: 10.1093/pcp/pcv064.

Oh, Y., Donofrio, N., Pan, H., Coughlan, S., Brown, D.E., Meng, S., et al. (2008). Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biology* 9**,** 1-24. doi: 10.1186/gb-2008-9-5-r85.

Pegg, G.S., Giblin, F., Mctaggart, A., Guymer, G., Taylor, H., Ireland, K., et al. (2014). *Puccinia psidii* in Queensland, Australia: Disease symptoms, distribution and impact. *Plant Pathology* 63**,** 1005-1021. doi: 10.1111/ppa.12173.

Pieterse, C.M.J., Leon Reyes, H.A., Van Der Ent, S., and Van Wees, A.C.M. (2009). Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* 5**,** 308-316. doi: 10.1038/nchembio.164.

Pieterse, C.M.J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C.M. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology* 28**,** 489-521. doi: 10.1146/annurev-cellbio-092910-154055.

Pitzschke, A., Schikora, A., and Hirt, H. (2009). MAPK cascade signalling networks in plant defence. *Current Opinion in Plant Biology* 12**,** 421-426. doi: 10.1016/j.pbi.2009.06.008.

Plotnikova, L.Y., Pozherukova, V.Y., Mitrofanova, O.P., and Degtyarev, A.I. (2016). The effect of oxidative burst suppression or induction on the interaction between brown rust fungus and timopheevi wheat. *Applied Biochemistry and Microbiology* 52**,** 61-70. doi: 10.1134/S0003683816010099.

Polonio, A., Seoane, P., Claros, M.G., and Perez-Garcia, A. (2019). The haustorial transcriptome of the cucurbit pathogen *Podosphaera xanthii* reveals new insights into the biotrophy and pathogenesis of powdery mildew fungi. *BMC Genomics* 20**,** 18. doi: 10.1186/s12864-019-5938-0.

Quecine, M.C., Leite, T.F., Bini, A.P., Regiani, T., Franceschini, L.M., Budzinski, I.G.F., et al. (2016). Label-free quantitative proteomic analysis of *Puccinia psidii* uredospores reveals differences of fungal populations infecting *Eucalyptus* and guava. *PLoS One* 11**,** e0145343. doi: 10.1371/journal.pone.0145343.

Rahman, M., Madina, M.H., Plourde, M.B., dos Santos, K.C.G., Huang, X., Zhang, Y., et al. (2021). The fungal effector Mlp37347 alters plasmodesmata fluxes and enhances susceptibility to pathogen. *Microorganisms* 9**,** 1232.

Rayachhetry, M.B., Elliott, M.L., and Van, T.K. (1997). Natural epiphytotic of the rust *Puccinia psidii* on *Melaleuca quinquenervia* in Florida. *Plant Disease* 81**,** 831. doi: 10.1094/pdis.1997.81.7.831a.

Ross-Davis, A.L., Graca, R.N., Alfenas, A.C., Peever, T.L., Hanna, J.W., Uchida, J.Y., et al. (Year). "Tracking the distribution of *Puccinia psidii* genotypes that cause rust disease on diverse Myrtaceous trees and shrubs", in: *In: Chadwick, K.; Palacios, P., comps. Proceedings of the 61st Annual Western International Forest Disease Work Conference; October 6-11, 2013; Waterton Lakes National Park; AB, Canada. Washington, DC: US Department of Agriculture, Forest Service, Forest Health Protection. p. 131-137.*), 131-137.

Roux, J., Germishuizen, I., Nadel, R., Lee, D.J., Wingfield, M.J., and Pegg, G.S. (2015). Risk assessment for *Puccinia psidii* becoming established in South Africa. *Plant Pathology* 64**,** 1326-1335. doi: 10.1111/ppa.12380.

Roux, J., Granados, G., Shuey, L., Barnes, I., Wingfield, M.J., and Mctaggart, A.R. (2016). A unique genotype of the rust pathogen, *Puccinia psidii,* on Myrtaceae in South Africa. *Australasian Plant Pathology* 45**,** 645-652. doi: 10.1007/s13313-016-0447-y.

Roux, J., Greyling, I., Coutinho, T.A., Verleur, M., and Wingfield, M.J. (2013). The Myrtle rust pathogen, *Puccinia psidii*, discovered in Africa. *IMA fungus* 4**,** 155-159. doi: 10.5598/imafungus.2013.04.01.14.

Ruan, J., Zhou, Y., Zhou, M., Yan, J., Khurshid, M., Weng, W., et al. (2019). Jasmonic acid signaling pathway in plants. *International Journal of Molecular Sciences* 20**,** 2479. doi: 10.3390/ijms20102479.

Sacristan, S., and García-Arenal, F. (2008). The evolution of virulence and pathogenicity in plant pathogen populations. *Molecular Plant Pathology* 9**,** 369-384. doi: 10.1111/j.1364-3703.2007.00460.X.

Saini, S., Sharma, I., and Pati, P.K. (2015). Versatile roles of brassinosteroid in plants in the context of its homoeostasis, signaling and crosstalks. *Frontiers in Plant Science* 6**,** 950. doi: 10.3389/fpls.2015.00950.

Salla, T.D., Astarita, L.V., and Santarém, E.R. (2016). Defense responses in plants of *Eucalyptus* elicited by *Streptomyces* and challenged with *Botrytis cinerea*. *Planta* 243**,** 1055-1070. doi: 10.1007/s00425-015-2460-8.

Santos, S.A., Vidigal, P.M.P., GuimarãEs, L.M.S., Mafia, R.G., Templeton, M.D., and Alfenas, A.C. (2020). Transcriptome analysis of *Eucalyptus grandis* genotypes reveals constitutive overexpression of genes related to rust (*Austropuccinia psidii*) resistance. *Plant Molecular Biology*. doi: 10.1007/s11103-020-01030-x.

Sekiya, A., Marques, F.G., Leite, T.F., Cataldi, T.R., De Moraes, F.E., Pinheiro, A.L.M., et al. (2021). Network analysis combining proteomics and metabolomics reveals new insights into early responses of *Eucalyptus grandis* during rust infection. *Frontiers in Plant Science* 11. doi: 10.3389/fpls.2020.604849.

Shepherd, M., Bartle, J., Lee, D.J., Brawner, J., Bush, D., Turnbull, P., et al. (2011). Eucalypts as a biofuel feedstock. *Biofuels* 2**,** 639-657. doi: 10.4155/bfs.11.136.

Silva, E., Da Graça José, P., Porto, C., Martin Do Prado, R., Hoffmann-Campo, C.B., Meyer, M.C., et al. (2020a). Unraveling Asian soybean rust metabolomics using mass spectrometry and molecular Networking approach. *Scientific Reports (Nature Publisher Group)* 10. doi: 10.1038/s41598-019-56782-4.

Silva, R.R., Da Silva, A.C., Rodella, R.A., Marques, M.O.M., Zanuncio, A.J.V., Soares, M.A., et al. (2020b). Limonene, a chemical compound related to the resistance of *Eucalyptus* species to *Austropuccinia psidii*. *Plant Disease* 104**,** 414-422. doi: 10.1094/pdis-05-19-1002-re.

Slippers, B., Stenlid, J., and Wingfield, M.J. (2005). Emerging pathogens: Fungal host jumps following anthropogenic introduction. *Trends in Ecology & Evolution* 20**,** 420-421. doi: 10.1016/j.tree.2005.05.002.

Soewarto, J., Giblin, F., and Carnegie, A.J. (2019a). *Austropuccinia psidii (myrtle rust) global host list. Version 4* [Online]. Canberra, ACT: Australian Network for Plant Conservation. http://www.anpc.asn.au/myrtle-rust. Accessed 20 November 2020. Available: http://www.anpc.asn.au/myrtle-rust [Accessed 20 November 2020].

Soewarto, J., Hamelin, C., Bocs, S., Mournet, P., Vignes, H., Berger, A., et al. (2019b). Transcriptome data from three endemic Myrtaceae species from New Caledonia displaying contrasting responses to myrtle rust (*Austropuccinia psidii*). *Data in Brief* 22**,** 794-811. doi: 10.1016/j.dib.2018.12.080.

Stewart, J.E., Ross-Davis, A.L., Graça, R.N., Alfenas, A.C., Peever, T.L., Hanna, J.W., et al. (2018). Genetic diversity of the myrtle rust pathogen (*Austropuccinia psidii*) in the Americas and Hawaii: Global implications for invasive threat assessments. *Forest Pathology* 48**,** e12378. doi: 10.1111/efp.12378.

Swanepoel, S., Oates, C.N., Shuey, L.S., Pegg, G.S., and Naidoo, S. (2021). Transcriptome analysis of *Eucalyptus grandis* implicates brassinosteroid signaling in defense against myrtle rust (*Austropuccinia psidii*). *Frontiers in Forests and Global Change* 4**,** 778611. doi: 10.3389/ffgc.2021.778611.

Tang, W., Yuan, M., Wang, R., Yang, Y., Wang, C., Oses-Prieto, J.A., et al. (2011). PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. *Nature Cell Biology* 13**,** 124-131. doi: 10.1038/ncb2151.

Thumma, B., Pegg, G.S., Warburton, P., Brawner, J.T., Macdonell, P., Yang, X., et al. (2013). Molecular tagging of rust resistance genes in eucalypts. 1-15.

Tobias, P.A., Guest, D.I., Külheim, C., and Park, R.F. (2018). De novo transcriptome study identifies candidate genes involved in resistance to *Austropuccinia psidii* (myrtle rust) in *Syzygium luehmannii* (riberry). *Phytopathology* 108**,** 627-640. doi: 10.1094/PHYTO-09-17-0298-R.

Tobias, P.A., Schwessinger, B., Deng, C.H., Wu, C., Dong, C., Sperschneider, J., et al. (2021). *Austropuccinia psidii,* causing myrtle rust, has a gigabase-sized genome shaped by transposable elements. *G3 Genes|Genomes|Genetics* 11**,** jkaa015. doi: 10.1093/g3journal/jkaa015.

Torres, M.A., Jones, J.D.G., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiology* 141**,** 373-378. doi: 10.1104/pp.106.079467.

Trujillo-Moya, C., Ganthaler, A., Stoggl, W., Kranner, I., Schuler, S., Ertl, R., et al. (2020). RNA-Seq and secondary metabolite analyses reveal a putative defence-transcriptome in Norway spruce (*Picea abies*) against needle bladder rust (*Chrysomyxa rhododendri*) infection. *BMC Genomics* 21**,** 21. doi: 10.1186/s12864-020-6587-z.

Uchida, J., Zhong, S., and Killgore, E. (2006). First report of a rust disease on Ohia caused by *Puccinia psidii* in Hawaii. *Plant Disease* 90**,** 524. doi: 10.1094/pd-90-0524c.

Ullah, C., Tsai, C.J., Unsicker, S.B., Xue, L.J., Reichelt, M., Gershenzon, J., et al. (2019). Salicylic acid activates poplar defense against the biotrophic rust fungus *Melampsora larici-populina* via increased biosynthesis of catechin and proanthocyanidins. *New Phytologist* 221**,** 960-975. doi: 10.1111/nph.15396.

Urban, M., Irvine, A., Cuzick, A., and Hammond-Kosack, K. (2015). Using the pathogen-host interactions database (PHI-base) to investigate plant pathogen genomes and genes implicated in virulence. *Frontiers in Plant Science* 6**,** 605. doi: 10.3389/fpls.2015.00605.

Van De Wouw, A.P., and Howlett, B.J. (2011). Fungal pathogenicity genes in the age of 'omics'. *Molecular Plant Pathology* 12**,** 507-514. doi: 10.1111/j.1364-3703.2010.00680.x.

Van Der Biezen, E.A., and Jones, J.D.G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23**,** 454-456. doi: 10.1016/S0968-0004(98)01311-5.

Van Der Hoorn, R.a.L., and Kamoun, S. (2008). From guard to decoy: A new model for perception of plant pathogen effectors. *The Plant Cell* 20**,** 2009-2017. doi: 10.1105/tpc.108.060194.

Van Loon, L.C., Bakker, P.A., and Pieterse, C.M.J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36**,** 453-483. doi: 10.1146/annurev.phyto.36.1.453.

Van Loon, L.C., Rep, M., and Pieterse, C.M.J. (2006). Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* 44**,** 135-162. doi: 10.1146/annurev.phyto.44.070505.143425.

Verma, V., Ravindran, P., and Kumar, P.P. (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biol* 16**,** 1-10. doi: 10.1186/s12870-016-0771-y.

Villalta, O.N., Mebalds, M., and Edwards, J. (2018). Biosecurity tool-set: optimising quarantine zones for eradication through consideration of Australia's success with plant pathogen eradications in horticulture. *Australasian Plant Pathology* 47**,** 135-154. doi: 10.1007/s13313-018-0551-2.

Voegele, R.T., Hahn, M., Lohaus, G., Link, T., Heiser, I., and Mendgen, K. (2005). Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. *Plant Physiology* 137**,** 190-198. doi: 10.1104/pp.104.051839.

Voegele, R.T., and Mendgen, K. (2003). Rust haustoria: Nutrient uptake and beyond. *New Phytologist* 159**,** 93-100. doi: 10.1046/j.1469-8137.2003.00761.x.

Wahl, R., Wippel, K., Goos, S., Kämper, J., and Sauer, N. (2010). A novel high-affinity sucrose transporter is required for virulence of the plant pathogen *Ustilago maydis*. *PLoS Biology* 8**,** e1000303. doi: 10.1371/journal.pbio.1000303.

Wang, X., and Chory, J. (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signaling, from the plasma membrane. *Science* 313**,** 1118-1122. doi: 10.1126/science.1127593.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., et al. (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Developmental Cell* 15**,** 220-235. doi: 10.1016/j.devcel.2008.06.011.

Wang, X., Yang, B., Li, K., Kang, Z., Cantu, D., and Dubcovsky, J. (2016). A conserved *Puccinia striiformis* protein interacts with wheat NPR1 and reduces induction of *pathogenesis-related* genes in response to pathogens. *Molecular Plant-Microbe Interactions* 29**,** 977-989. doi: 10.1094/MPMI-10-16-0207-R.

Wasternack, C., and Hause, B. (2013). Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Ann. Bot.* 111**,** 1021-1058. doi: 10.1093/aob/mct067.

Westermann, A.J., Gorski, S.A., and Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nature Reviews Microbiology* 10**,** 618-630. doi: 10.1038/nrmicro2852.

Wingfield, M.J., Brockerhoff, E.G., Wingfield, B.D., and Slippers, B. (2015). Planted forest health: The need for a global strategy. *Science* 349**,** 832-836. doi: 10.1126/science.aac6674.

Wingfield, M.J., Roux, J., Slippers, B., Hurley, B.P., Garnas, J., Myburg, A.A., et al. (2013). Established and new technologies reduce increasing pest and pathogen threats to Eucalypt plantations. *Forest Ecology and Management* 301**,** 35-42. doi: 10.1016/j.foreco.2012.09.002.

Wingfield, M.J., Slippers, B., Hurley, B.P., Coutinho, T.A., Wingfield, B.D., and Roux, J. (2008). Eucalypt pests and diseases: Growing threats to plantation productivity. *Southern Forests: A Journal of Forest Science* 70**,** 139-144. doi: 10.2989/SOUTH.FOR.2008.70.2.9.537.

Winter, G. (1884). Repertorium. Rabenhorstii fungi europaei et extraeuraopaei. Cent. XXXI et XXXII. *Hedwigia* 23**,** 164-172.

Wu, W., Nemri, A., Blackman, L.M., Catanzariti, A.-M., Sperschneider, J., Lawrence, G.J., et al. (2019). Flax rust infection transcriptomics reveals a transcriptional profile that may be indicative for rust Avr genes. *PloS one* 14**,** e0226106.

Wu, Y., Zhang, D., Chu, Jee y., Boyle, P., Wang, Y., Brindle, Ian d., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports* 1**,** 639-647. doi: 10.1016/j.celrep.2012.05.008.

Xavier, A.A., Alfenas, A.C., Matsuoka, K., and Hodges, C.S. (2001). Infection of resistant and susceptible *Eucalyptus grandis* genotypes by urediniospores of *Puccinia psidii*. *Australasian Plant Pathology* 30**,** 277-281. doi: 10.1071/ap01038.

Xavier, A.A., Da Silva, A.C., Da Silva Guimarães, L.M., Matsuoka, K., Hodges, C.S., and Alfenas, A.C. (2015). Infection process of *Puccinia psidii* in *Eucalyptus grandis* leaves of different ages. *Tropical Plant Pathology* 40**,** 318-325. doi: 10.1007/s40858-015-0043-7.

Xu, Q., Tang, C., Wang, L., Zhao, C., Kang, Z., and Wang, X. (2020). Haustoria–arsenals during the interaction between wheat and *Puccinia striiformis* f. sp. *tritici*. *Molecular Plant Pathology* 21**,** 83-94. doi: 10.1111/mpp.12882.

Yang, L., Wen, K.-S., Ruan, X., Zhao, Y.-X., Wei, F., and Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. *Molecules* 23**,** 762. doi: 10.3390/molecules23040762.

Yong, W.T.L., Ades, P.K., Runa, F.A., Bossinger, G., Sandhu, K.S., Potts, B.M., et al. (2021). Genome-wide association study of myrtle rust (*Austropuccinia psidii*) resistance in *Eucalyptus obliqua* (subgenus *Eucalyptus*). *Tree Genetics & Genomes* 17. doi: 10.1007/s11295-021-01511-0.

Yong, W.T.L., Ades, P.K., Tibbits, J.F.G., Bossinger, G., Runa, F.A., Sandhu, K.S., et al. (2019). Disease cycle of *Austropuccinia psidii* on *Eucalyptus globulus* and *Eucalyptus obliqua* leaves of different rust response phenotypes. *Plant Pathology* 68**,** 547-556. doi: 10.1111/ppa.12959.

Yu, M.-H., Zhao, Z.-Z., and He, J.-X. (2018). Brassinosteroid signaling in plant-microbe interactions. *International Journal of Molecular Sciences* 19**,** 4091. doi: 10.3390/ijms19124091.

Yu, X., Feng, B.M., He, P., and Shan, L.B. (2017). From chaos to harmony: responses and signaling upon microbial pattern recognition. *Annual Review of Phytopathology* 55**,** 109-137. doi: 10.1146/annurev-phyto-080516-035649.

Zauza, E.a.V., Lana, V.M., Maffia, L.A., Araujo, M.M.F.C., Alfenas, R.F., Silva, F.F., et al. (2015). Wind dispersal of *Puccinia psidii* urediniospores and progress of eucalypt rust. *Forest Pathology* 45**,** 102-110. doi: 10.1111/efp.12133.

Zhang, Y., and Li, X. (2019). Salicylic acid: Biosynthesis, perception, and contributions to plant immunity. *Current Opinion in Plant Biology* 50**,** 29-36. doi: 10.1016/j.pbi.2019.02.004.

Zhang, Y., Tessaro, M.J., Lassner, M., and Li, X. (2003). Knockout analysis of Arabidopsis transcription factors *TGA2*, *TGA5*, and *TGA6* reveals their redundant and essential roles in systemic acquired resistance. *The Plant Cell* 15**,** 2647-2653. doi: 10.1105/tpc.014894.

Zhuang, J., and Wei, S. (2011). Additional materials for the rust flora of Hainan Province, China. *Mycosystema* 30**,** 853-860.

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends in Immunology* 35**,** 345-351. doi: 10.1016/j.it.2014.05.004.

1.10 Figures

Figure 1.1. The global distribution of the myrtle rust pathogen, *Austropuccinia psidii*, showing reportedly affected countries. Blue shaded regions represent affected countries while grey shaded regions represent unaffected countries. Central and South America, depicted by the red box, are the proposed origins of myrtle rust. Data was compiled from https://www.cabi.org/isc/datasheet/45846 (accessed July 2020).

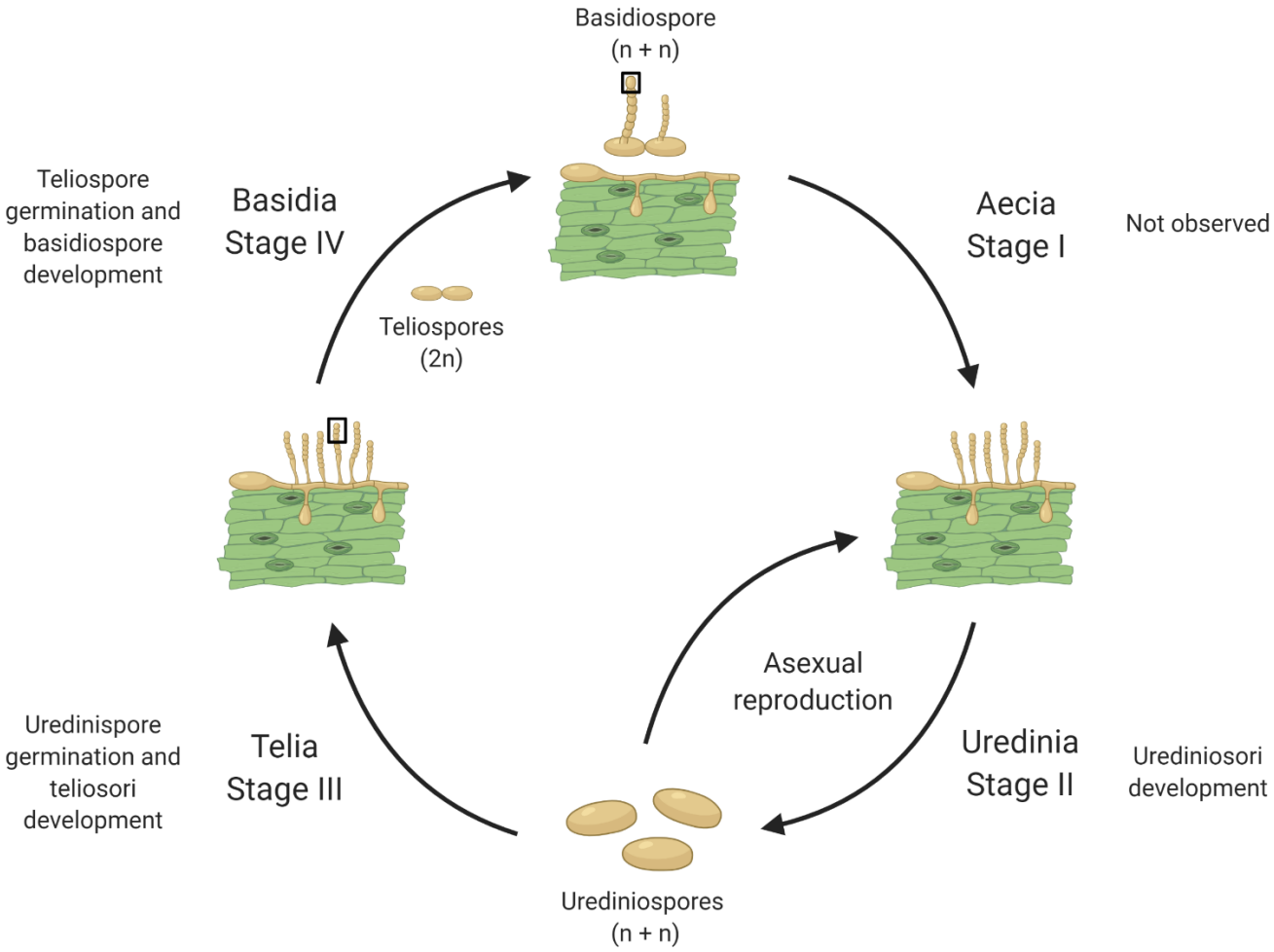

Figure 1.2. The lifecycle of the macrocyclic, autoecious rust fungus *Austropuccinia psidii* showing the four known stages. Urediniospores can undergo asexual reproduction in which the urediniospores inoculate host plants, undergo germination, leading to urediniosori develop. These mature to urediniospores that can re-infect the host or travel via wind to infect other host plants. Adapted from Glen et al. (2007), Bakkeren and Szabo (2020) and created with BioRender.com.

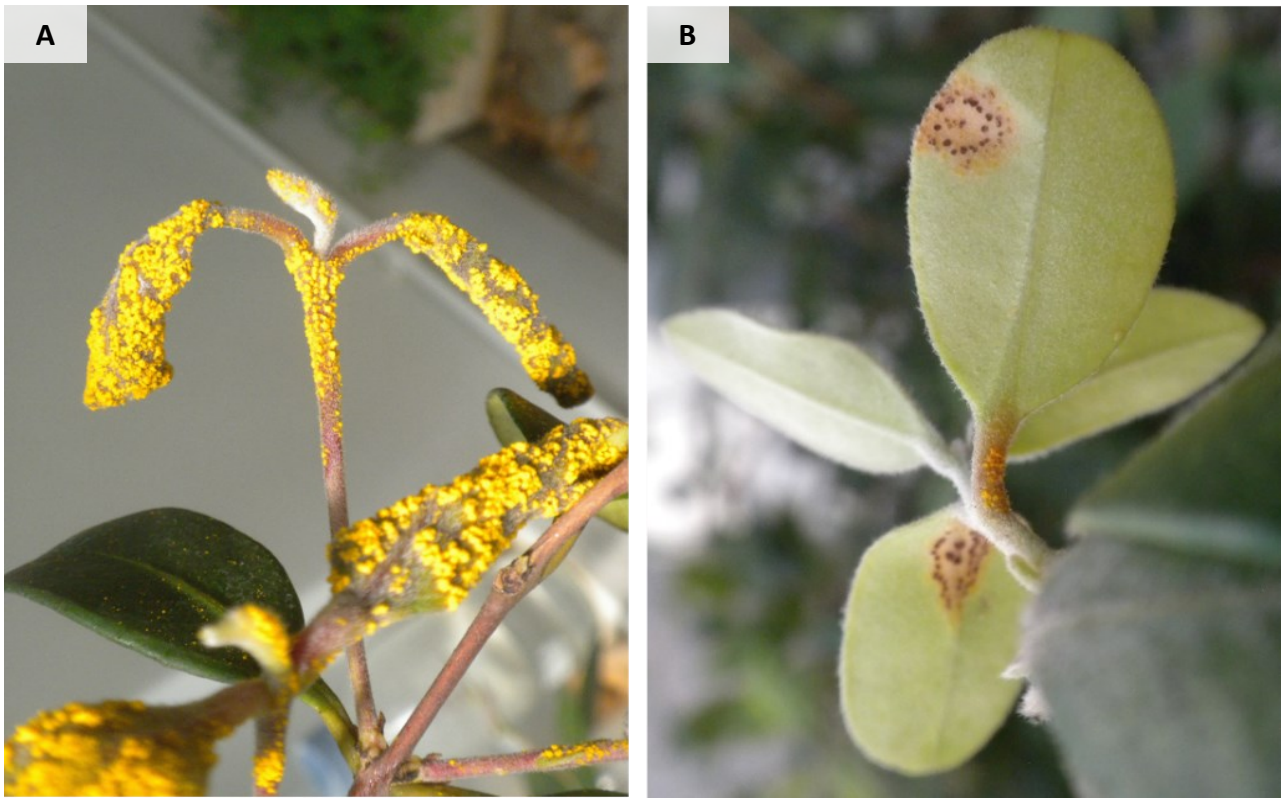

Figure 1.3. The symptoms of myrtle rust infection caused by *Austropuccinia psidii* on *Eugenia urophylla* native to South Africa, showing the two visible spore stages. (A) The characteristic yellow urediniospores and (B) the brown lesions caused by the teliospore stage of *Austropuccinia psidii* (photographs by L. Shuey, unpublished).

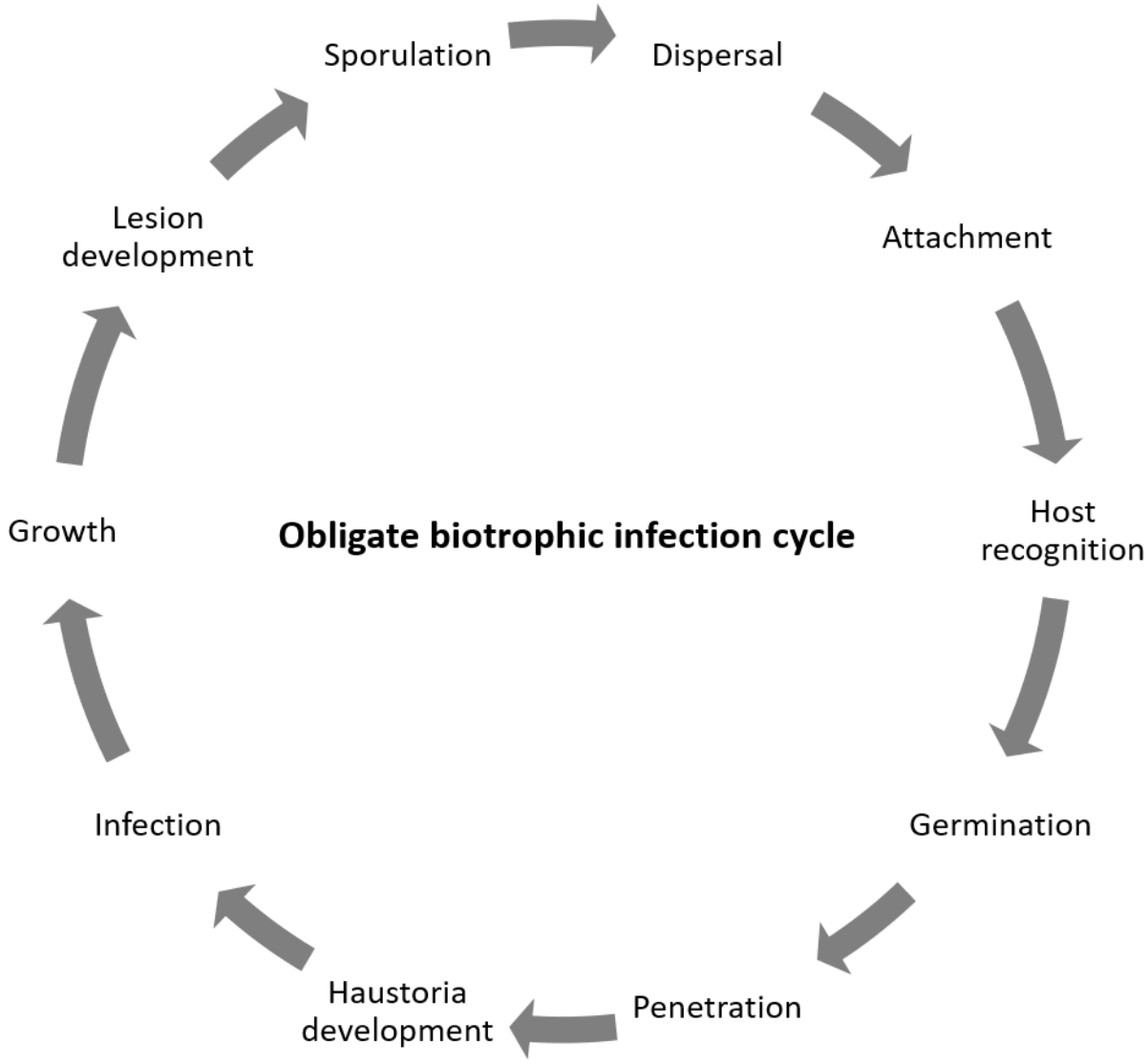

Figure 1.4. A schematic diagram of a typical infection cycle. The cycle begins when a wind-borne spore attaches to a host plant. Once the spore recognises the host, germination begins in which an appressorium forms. From the appressorium, a penetration peg emerges that allows the fungus to gain access to the host cells. This is followed by haustoria development from which effectors and toxic metabolites are secreted to acquire host-derived nutrients and suppress host immune responses. This leads to hyphal development and subsequent infection, in which the host defence responses are successfully overcome. The pathogen continues to develop and grow within the host plant, acquiring nutrients and suppressing immunity until a lesion develops. From the lesion on the host surface, sporulation of new spores occurs that are ready for dispersal to new or the same host. Figure adapted from Meng et al. (2009).

Chapter 2

Research Chapter

Transcriptome analysis of *Eucalyptus grandis* implicates brassinosteroid signalling in defence against myrtle rust (*Austropuccinia psidii*)

Shae Swanepoel [1], Caryn N. Oates [1], Louise S. Shuey [2], Geoff Pegg [2], Sanushka Naidoo [1]

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

[2] Department of Agriculture and Fisheries, Queensland Government, Australia

This chapter has been prepared in the format of a manuscript for a peer-reviewed research journal and has been published in Frontiers in Forests and Global Change. Louise Shuey and Geoff Pegg collected the sample material. I analysed the data, drafted the manuscript, and prepared the figures. Caryn Oates and Geoff Pegg helped draft the manuscript. Sanushka Naidoo and Louise Shuey conceived of and supervised the study as well as helped draft the manuscript.

2.1 Abstract

Eucalyptus grandis, in its native Australian range, varies in resistance to *Austropuccinia psidii (*syn. *Puccinia psidii)*. The biotrophic rust fungus, *A. psidii* is the causal agent of myrtle rust and poses a serious threat to Australian biodiversity. The pathogen produces yellow pustules of urediniospores on young leaves and shoots, resulting in shoot tip dieback, stunted growth, and death. Dissecting the underlying mechanisms of resistance against this pathogen will contribute to improved breeding and control strategies to mitigate its devastating effects. The aim of this study was to determine the molecular dialogue between *E. grandis* and *A. psidii*, using an RNA-sequencing approach. Resistant and susceptible *E. grandis* seedlings grown from seed collected across its natural range were inoculated with the pandemic biotype of *A. psidii*. The leaf tissue was harvested at 12-hours post inoculation (hpi), 1-day post inoculation (dpi), 2-dpi and 5-dpi and subjected to RNA-sequencing using Illumina 50bp PE reads to a depth of 40 million reads per sample. Differential gene expression and gene ontology enrichment indicated that the resistant seedlings showed controlled, coordinated responses with a hypersensitive response, while the susceptible seedlings showed no systemic response against myrtle rust. Brassinosteroid signalling was apparent as an enriched term in the resistant interaction at 2-dpi, suggesting an important role of this phytohormone in defence against the pathogen. Brassinosteroid mediated signalling genes were also among the candidate genes within two major disease resistance loci (*Puccinia psidii* resistance), *Ppr3* and *Ppr5.* While brassinosteroids have been tagged as positive regulators in other plant disease resistance interactions, this is the first report in the *Eucalyptus – Austropuccinia psidii* interaction. Furthermore, several putative resistance genes, underlying known resistance loci and implicated in the interaction have been identified and highlighted for future functional studies. This study provided further insights into the molecular interactions between *E. grandis* and *A. psidii*, contributing to our understanding of this pathosystem.

Keywords: *Austropuccinia psidii, Eucalyptus,* RNA-seq, phytohormones, resistance loci

2.2 Introduction

Austropuccinia psidii (G. Winter) Beenken (formerly *Puccinia psidii*, commonly myrtle rust) is a biotrophic fungal pathogen causing widespread devastation to the natural and commercial range of numerous plant species within the Myrtaceae family. Originating in South America, this pathogen jumped from native Myrtaceae to introduced *Eucalyptus* and has since spread across the world causing devastation to natural and introduced Myrtaceae species (Carnegie and Pegg, 2018). Currently, *A. psidii* has been discovered in South and Central America (Coutinho et al., 1998) and Florida, California and Hawaii in the USA, North America (Marlatt and Kimbrough, 1979; Rayachhetry et al., 1997; Uchida et al., 2006). The pathogen has also been reported in countries such as Japan (Kawanishi et al., 2009), Australia (Carnegie et al., 2010), China (Zhuang and Wei, 2011), South Africa (Roux et al., 2013), New Caledonia (Giblin, 2013), Indonesia (McTaggart et al., 2016), Singapore and New Zealand (du Plessis et al., 2019). Myrtle rust has an unusually broad host range on various members of the Myrtaceae family, affecting approximately 480 species within 86 genera, producing yellow pustules of urediniospores on growing leaves and shoots (Coutinho et al., 1998; Soewarto et al., 2019). This results in dieback of shoot tips, stunted growth and in severe cases plant death (Carnegie et al., 2016; Pegg et al., 2017; Fensham et al., 2020; Pegg et al., 2020). *Eucalyptus* spp. are amongst the most susceptible members of this family to myrtle rust, posing a risk to their natural and introduced ranges (Coutinho et al., 1998; Junghans et al., 2003a), with *Eucalyptus grandis* showing variable responses ranging from highly susceptible to resistant.

Plants have evolved complex and integrated defence mechanisms against pathogenic organisms, including preformed and induced defences. Preformed defences include factors such as cuticle waxes on the leaf surface, cell walls and secondary metabolites (Naidoo et al., 2014). dos Santos *et al.* (2019) found the chemical composition of the cuticle waxes in *E. grandis* and *E. phaeotricha* contribute significantly to susceptibility against *A. psidii*, with various cuticular signals influencing the interaction between host and pathogen. Additionally, older leaves of *E. grandis* have been shown to comprise greater amounts of waxes than younger leaves which may affect the ability of *A. psidii* urediniospores to adhere to or penetrate the leaf surface. This results in pre-penetration resistance in mature trees against this pathogen (Xavier et al., 2015). When a urediniospore successfully attaches to the surface of the leaf, the spore germinates and develops a germ tube. From the germ tube, an appressorium forms which gives rise to a penetration peg that infiltrates the cuticle (Coutinho et al., 1998). Following leaf infiltration, haustoria develop in the host cells (Coutinho et al., 1998; Glen et al., 2007). It is through these haustoria that the pathogen acquires nutrients from the host plant to ensure its survival (Hahn

and Mendgen, 2001) and sustained release of proteins that suppress the host defence responses (Jones and Dangl, 2006). These proteins are known as effectors (or avirulence proteins) that contribute to the success of the pathogen (Jones and Dangl, 2006).

Junghans et al. (2003a) identified a locus associated with *A. psidii* resistance in *E. grandis*, termed *Puccinia psidii resistance 1* (*Ppr1*), as per the previous classification of *A. psidii*. *Ppr1* was shown to contribute to a large portion of resistance against *A. psidii*. This locus was found to be associated with numerous nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance genes (*R*-genes) (Thumma et al., 2013), which suggests resistance against *A. psidii* is controlled by a combination of these genes. The discovery of this locus allowed targeted breeding programmes, selecting for rust resistance to combat this pathogen. Despite the significance of *Ppr1* in rust resistance, clones carrying this locus were shown to be susceptible to certain biotypes of *A. psidii*, indicating the locus was failing at providing resistance against *A. psidii* (Graça et al., 2011). Four additional loci contributing to myrtle rust resistance were discovered in *E. globulus* (Butler et al., 2016), termed *Ppr2, Ppr3, Ppr4* and *Ppr5.* These quantitative trait loci (QTLs) determine if the plant shows symptoms (*Ppr2* and *Ppr3*) and if there is a hypersensitive response (HR, *Ppr4* and *Ppr5*) (Butler et al., 2016). The identification of additional QTLs contributing to disease resistance highlights the complexity of rust resistance. Despite the significance of these resistance loci, few studies have investigated the expression patterns of genes underlying these loci and little is known about how these genes contribute to disease resistance.

A recent study aimed to investigate the transcriptome of *Melaleuca quinquenervia* upon infection by *A. psidii,* to identify differences in responses among individuals with varying responses to the pathogen (Hsieh et al., 2018). At 5-dpi, they found that susceptible plants had higher levels of fungal transcripts than resistant plants, suggesting *A. psidii* could grow more successfully in susceptible leaves. Additionally, the authors found numerous differentially expressed genes (DEGs) associated with defence. These include *NBS-LRR* genes, *WRKY* transcription factors and *Pathogenesis-related* (*PR*) genes, such as *PR-1, PR-2, PR-3, PR-4, and PR-5*, with expression varying among resistant individuals. The authors suggest a potential role of the phenylpropanoid pathway in resistance against *A. psidii*, however, expression of associated genes differed among resistant samples and further studies are required to confirm this involvement. Sekiya et al. (2021), studying the interaction between *E. grandis* and *A. psidii*, found that rust resistance largely depends on the control of phenylpropanoid pathway related enzymes, corroborating the involvement of this pathway in defence against *A. psidii*. Tobias et al. (2018) studied the transcriptional responses of *Syzygium luehmannii* challenged by *A. psidii*. The authors found up-regulation of receptor-like kinase genes, *NBS-LRR* genes as well as genes encoding enzymes involved in secondary metabolism which may contribute to disease resistance.

Furthermore, they identified two TIR-NBS-LRR-type genes differentially expressed at 2-dpi with *E. grandis* homologs predicted within the *Ppr1* locus. Santos et al. (2020) investigated the transcriptomes of resistant and susceptible *E. grandis* upon infection with *A. psidii* after 24-hpi. The authors found constitutive overexpression of defence-related genes in resistant compared to susceptible genotypes. These responses included up-regulation of salicylic acid (SA) associated genes, signal transduction and protein kinase leucine-rich receptors (PK-LRR). These studies provide some of the first insights into the transcriptional responses of Myrtaceae species challenged by *A. psidii* and further studies of these interactions are required to gain a deeper understanding of the molecular mechanisms governing host resistance.

The present study aimed to investigate the molecular interaction of resistant and susceptible *Eucalyptus grandis* challenged by *A. psidii* over time. In susceptible *E. grandis* genotypes, mycelia and haustoria have reportedly been observed between 12- and 18-hpi and in resistant genotypes, these have been observed between 18-hpi and 1-dpi. Tobias et al. (2018) found significant transcriptome changes between resistant and susceptible samples 2-dpi whereas Hsieh et al. (2018) reported the possibility of fungal parasitic feeding stage at 5-dpi. Therefore, we hypothesize that at these and earlier time points (12-hpi, 1-, 2- and 5-dpi) investigation of the expression profiles of resistant and susceptible seedlings would reveal molecular responses governing resistance, including changes in phytohormones, reactive oxygen species (ROS) and resistance genes. Through this, we have identified putative pathways and genes that contribute to host defence in *E. grandis* that can be manipulated in breeding and control programmes, to mitigate the effects of myrtle rust. This is the first study to look at the transcriptome of *E. grandis* upon infection by *A. psidii* over a time course and provides further insights into the molecular interactions.

2.3 Materials and Methods

2.3.1 *Austropuccinia psidii* inoculation trial

Eucalyptus grandis seedlings were grown from seed sourced from wild plants across its natural distribution in eastern Australia, ranging from Coffs Harbour in New South Wales to northern tropical regions of Queensland. Seed was germinated in glasshouse conditions, where temperatures ranged from 20-30ºC. The seedlings, all with at least four young leaves, were inoculated to initially determine the phenotypes. Inoculations were generally carried out as described by Pegg et al. (2014). Spores of *A. psidii* were however collected from *Syzygium jambos* growing as street trees in suburbs of Brisbane, Queensland rather than using spores bulked from single spore inoculations in the laboratory. Spores

were shaken into paper bags and passed through a sieve in the laboratory to remove plant debris. The collected spores were then desiccated in silica gel for 48 h and stored at -80ºC. Urediniospores of the pandemic biotype were removed from -80°C storage and left at room temperature before addition of sterile distilled water with 0.05% tween 20. Spore were counted using a haemocytometer, with the concentration adjusted to 1×10^5 spore mL^{-1}. Six-week-old *E. grandis* seedlings were spray inoculated with *A. psidii* urediniospores using a fine mist at a pressure of 2.9 kPa. Spores were applied to the upper and lower leaf surfaces, and the seedling trays were covered with a plastic sheet for 24 h in the dark at 18°C. After 24 h, the plastic covers were removed, and the seedlings were assessed for resistance and susceptibility based on their responses at two weeks post inoculation. Leaves were rated on a scale from one to five, where rating one was considered resistant (R-interaction) and rating five was considered susceptible (S-interaction) based on the system used in Junghans et al. (2003b) (Table 2.1).

Seedlings rated R and S were selected and separated out from other seedlings, and diseased tissues were removed by pruning. The seedlings were left to reshoot and after eight weeks inoculation trials were repeated to obtain samples for RNA-seq. Seedlings were either inoculated as described above (infected), or mock-inoculated with sterile distilled water with 0.05% tween 20 (control). The youngest two sets of leaves of the infected and control plants were sampled as these are the most susceptible to *A. psidii* infection. Samples were collected from the seedlings at four time intervals namely, 12-hpi, 1-, 2- and 5-dpi with every time point sampled from the same individual. Three replicates per time point were collected, comprising 14 seedlings per replicate to account for the genetic diversity within the seedlings.

2.3.2 RNA extraction and sequencing

Leaf samples collected from infected and control groups were subsequently frozen in liquid nitrogen. Total RNA was extracted as described by Zeng and Yang (2002) and treated with Qiagen RNase-free DNase 1 enzyme (Qiagen Inc.). Following successful RNA extraction, the samples were purified using Qiagen RNeasy Mini Kit as per the manufacturer's instructions. To determine the concentration and quality of the extracted RNA, the samples were analysed using Bio-Rad Experion analyser. Purified RNA from three biological replicates were submitted to the Beijing Genomics Institute (BGI) for mRNA-sequencing using 50 bp paired-end Illumina HiSeq 2500 with an insert size of 300 bp and a sequencing depth of 40 million reads per sample.

2.3.3 Mapping and analysis of reads against *Eucalyptus grandis* v2.0 genome assembly

RNA-seq data were analysed using the Galaxy workspace (Goecks et al., 2010) and FASTQC v0.11.3 was used to assess read quality. Reads from the R- and S-interactions at each time point (both infected and control samples) were mapped to the *E. grandis* v2.0 reference genome (Myburg et al., 2014) using Bowtie 2 (Langmead and Salzberg, 2012), Tophat2 v2.0.14 (Trapnell et al., 2012) and featureCounts v1.5.0-p3 (Liao et al., 2013). Pathogen transcripts were removed for the purpose of this study and will be investigated in future studies using the *A. psidii* reference genome (Tobias et al., 2021).

Data was normalized and variance stabilized transformation was applied in DESeq2 (Love et al., 2014) and transcripts per kilobase million (TPM) in R v3.6.0 (R Core Team, 2018). DEGs were analysed using DESeq2 with filtering based on a two-fold change (>1.00 or <-1.00 $\log_2$ (fold change)) and a false discovery rate of $P < 0.001$. We compared changes in gene expression between resistant, control relative to resistant, infected (R-interaction) and susceptible, control relative to susceptible, infected (S-interaction) to determine the effect pathogen infection would have on each interaction. Additionally, we compared susceptible, control relative to resistant, control (S-C vs. R-C) to determine the background gene expression. Heatmaps were generated using the R package ComplexHeatmap v2.0.0 (Gu et al., 2016) using $\log_2$ (fold change) values.

2.3.4 Enrichment analysis

Identified DEGs were analysed for Gene Ontology (GO) enrichment over-representation in the category biological processes (BP), to identify biological pathways potentially involved in the interaction. Enrichment was calculated using Fisher's exact test and *p*-values were adjusted using the Benjamini and Hochberg (BH) method with a false discovery rate (FDR) of 0.05, with all calculations performed in R v3.6.0. Enrichment was determined separately for all time points as well as up- and down-regulated DEGs. Additionally, Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotation enrichment was performed on DEGs to identify enriched KEGG terms using the same method.

2.3.5 Identification of candidate genes underlying *Austropuccinia psidii* resistance loci

To identify candidate genes underlying *A. psidii* resistance QTLs (*Ppr1, Ppr2, Ppr3, Ppr4* and *Ppr5*), the physical positions of their flanking markers were obtained (Table 2.2) (Junghans et al., 2003a;

Mamani et al., 2010; Butler et al., 2016). Phytozome v12.1 was used to identify genes underlying these physical positions of each locus on its respective linkage group using the genome browser.

2.4 Results

2.4.1 *Eucalyptus grandis* disease progression in response to *Austropuccinia psidii*

Disease progression was monitored routinely for the presence of symptoms. Disease assessments were performed on the primary and secondary foliage (youngest two expanding pairs of leaves) to avoid the influence leaf age may have on disease severity levels. Based on symptom development, seedlings were assessed 14-dpi. Different responses were observed in *E. grandis* seedlings following inoculations as shown in Figure 2.1. Symptoms were similar to those observed in previous inoculations performed by Junghans et al. (2003b). This included light chlorotic flecking on seedlings rated one. Visible HR and necrotic lesion formation on seedlings rated two. Progression of necrosis and formation of uredinia pustules were observed in ratings three and four. Severe pustules were observed in seedlings rated five, with disease commonly occurring on the petiole and stem. For the purpose of this study, plants rated one were considered resistant (R) and those rated five on the severity scale considered susceptible (S), with samples harvested from these ratings for further differential gene expression analysis.

2.4.2 Differential gene expression in resistant and susceptible *Eucalyptus grandis*

To determine the effects pathogen infection would have on infected seedlings, we compared infected and control seedlings for both the R- and S-interactions across the time series. There were 4,681 significantly DEGs over the time course in the R-interaction, while there were 2,800 DEGs in the S-interaction over the time course (data not shown). During early infection at 12-hpi, there were 50 down- and 96 up-regulated genes in the R-interaction, while the S-interaction had 39 down- and 309 up-regulated genes (Figure 2.2A). The number of up-regulated genes began increasing at 1-dpi in both the interactions. Up-regulation of genes peaked in the R-interaction at 2-dpi with 2,221 genes and 5-dpi in the S-interaction, with 2,146 genes (Figure 2.2A). Basal gene expression in resistant and susceptible plants might contribute to disease phenotypes when a pathogen is introduced, as previously observed in the *A. psidii-E. grandis* pathosystem (Santos et al., 2020). To investigate this, we compared susceptible, control relative to resistant, control (S-C vs. R-C) (Figure 2.2B). There was a total of 209 DEGs across the time course (data not shown). These results suggest that differences in basal expression exist.

To identify pathways putatively involved in resistance and susceptibility against *A. psidii*, GO enrichment was performed. No over-represented GO terms were identified in S-C vs. R-C, which is expected due to the low numbers of DEGs. Figure 2.3 shows a heatmap of over-represented defence-related terms in both the R- and S-interaction over the time series. Defence-related terms were separated into terms associated with phytohormone signalling and responses, oxidative burst, secondary metabolism and defence responses. Additionally, up- and down-regulated terms are represented. Terms enriched in the R-interaction involve up-regulation of genes associated with brassinosteroid (BR) signalling, at 2-dpi, genes associated with a range of salicylic acid (SA) associated pathways as well as jasmonic acid (JA) and ethylene (ET) associated pathway. Enriched terms in the S-interaction involve up-regulation of genes associated with JA and abscisic acid (ABA) pathways. Interestingly, the R-interaction showed up-regulation of secondary metabolic pathways including terpenoid transport, while these responses were absent/down-regulated in the S-interaction. Moreover, defence responses such as callose deposition were up-regulated in the R-interaction, while these responses were limited in the S-interaction.

The DEGs identified in the R- and S-interaction were analysed with KEGG to further elucidate the putative role these genes play in defence pathways. KEGG was used to determine pathways involved in defence against this pathogen. KEGG enrichment revealed up-regulation of "photosynthesis – antenna proteins" at 12-hpi, up-regulation of "terpenoid backbone biosynthesis" at 1-dpi and up-regulation of "amino sugar and nucleotide sugar metabolism" at 2-dpi unique to the R-interaction. Shared responses included plant-pathogen interaction at 1-dpi, 2-dpi, and 5-dpi. Interestingly, at 5-dpi, the S-interaction showed down-regulation of cutin, suberin and wax biosynthesis (Figure 2.4).

2.4.3 Brassinosteroid signalling in response to *Austropuccinia psidii* infection

In the R-interaction, BR signalling was observed at 2-dpi, with these responses absent in the S-interaction (Figure 2.3). Further investigations into the genes contributing to BR signalling revealed DEGs in both interactions, with the R-interaction expressing more of these genes at greater intensities. Signalling genes included BRI1-associated receptor kinase (*BAK1*), leucine-rich repeat protein kinase family proteins and *BZR1* family proteins. These genes showed different expression patterns across the R- and S-interaction over time, with the greatest expression observed in the R-interaction at 2-dpi. Similar responses were observed at 5-dpi in the S-interaction, although these terms were not enriched in the GO analysis (Figure 2.5).

2.4.4 Expression of candidate genes underlying *Austropuccinia psidii* resistance loci

To identify candidate genes underlying known resistance loci, the physical positions of the flanking markers were identified (Table 2.2) and the genes between the markers were selected for further investigation (Supplementary Table 2.1). Table 2.3 shows the total number of genes identified within each locus, with the number of DEGs underlying these loci. Additionally, the number of *R*-genes and differentially expressed (DE) *R*-genes underlying these loci are shown (Table 2.3, Supplementary Table 2.2). To determine the putative biological pathways that underlie the *A. psidii* resistance QTLs, the candidate genes identified were investigated for GO biological processes functional characterisation (Supplementary Figure 2.1). Defence-related enriched terms included "response to salicylic acid stimulus", "defence response" and "salicylic acid metabolic process". The involvement of these *Ppr* loci in defence-related processes highlighted throughout literature, the results of the GO BP enrichment analysis as well as the identification of numerous *R*-genes prompted further investigations into the expression profiles of the candidate genes underlying these QTLs.

To determine the roles the *A. psidii* resistance loci play in defence, DEGs underlying the QTLs were identified in the R-, S-interactions (Figure 2.6) and S-C vs. R-C (Figure 2.7A-I). Across all comparisons, there was significant differential expression of *R*-genes, namely *NBS-LRR* genes (Table 2.3, Supplementary Table 2.3). Figure 2.6 is a heatmap representing $\log_2$ (fold change) values of the DE *R*-genes underlying these QTLs in the R- and S-interactions. Differential expression of these *R*-genes is greater in the R-interaction, with up-regulation of numerous genes earlier in the interaction between 1- and 2-dpi when compared to the S-interaction. The S-interaction shows similar differential expression patterns later in the time series, between 2- and 5-dpi. The DE *R*-genes underlying *Ppr1, Ppr2, Ppr3* and *Ppr5* had low TPM values, despite showing significant differential expression. Interestingly, *Ppr4*, a locus determining if the plant exhibits HR (Butler et al., 2016), showed increased expression of these *R*-genes, with expression of these genes greater than those observed at the other disease resistance QTLs. This corroborates the findings in Figure 2.3, where HR-associated responses were significantly enriched in both the R- and S-interactions.

To determine the differences in constitutive expression of the *R*-genes underlying the disease resistance loci, we analysed S-C relative to R-C and found nine DE *R*-genes underlying these loci in the control samples (Table 2.3, Supplementary Table 2.3). Figure 2.7A-I shows the expression (TPM) of these genes over the time series. Eucgr.F01132 (Figure 2.7G) and Eucgr.F01139 (Figure 2.7H), underlying *Ppr4*, are highly expressed, this is similar to the findings in the R- and S-interactions, in which *R*-genes were highly expressed. Eucgr.I00210 (Figure 2.7I), underlying *Ppr5*, showed greater constitutive

expression in R-C at 12-hpi, 1- and 2-dpi, suggesting a role in constitutive resistance against *A. psidii*. Interestingly, Eucgr.C01921 (Figure 2.7B) underlying *Ppr1* showed significantly greater DE in R-C at 2-dpi than in S-C. Gene Eucgr.C02650 (Figure 2.7F) showed greater expression in S-C at 12-hpi, with the expression of this gene increasing R-C at 5-dpi. The timing and intensity of basal expression of these genes may contribute to the phenotypes observed when resistant and susceptible *E. grandis* is challenged by *A. psidii*. Further studies into the functional role of these genes are required to determine if they contribute to rust resistance and how these genes differ at the molecular level between resistant and susceptible seedlings.

Genes involved in BR mediated signalling was significantly over-represented in the R-interaction at 2-dpi (Figure 2.3 and Figure 2.5). To determine if the *Ppr* loci harbour genes associated with BR signalling, genes associated with this pathway identified through GO (GO:0009742) were compared to genes underlying the resistance loci. No BR mediated signalling genes were identified within *Ppr1, Ppr2* and *Ppr4.* Two and four BR mediated signalling genes were identified within *Ppr3* and *Ppr5,* respectively. These genes include a signalling kinase (BSK1), leucine-rich receptors (BRI1), protein kinases and a BR receptor (Supplementary Table 2.4).

2.5 Discussion

The aim of this study was to compare the transcriptional responses of resistant and susceptible *E. grandis* with *A. psidii* over time. Through this, we identified pathways that may be involved in the *E. grandis* defence response against myrtle rust, such as the hypersensitive response, oxidative burst, and a range of phytohormones, with brassinosteroids signalling identified in resistant seedlings. Additionally, the timing of these responses varied between R- and S-seedlings, with delays and absences of responses in susceptible hosts. This suggests the importance of a coordinated, rapid response in resistance against *A. psidii*. Analysis of loci associated with resistance revealed these resistance loci harbour a plethora of *R*-genes, and we have identified candidate *R*-genes that are DE in the interactions, showing potential involvement in the disease outcome. Overall, we suggest a combination of these responses contribute to the resistance observed in *E. grandis*.

2.5.1 Selection of resistant and susceptible seedlings

Despite the absence of physical symptoms of HR in the resistant seedlings, HR was significantly enriched in the resistant RNA-seq data, suggesting that this response is occurring at a molecular level. The plant material sourced for this study were seedlings rather than clones. This introduces genetic diversity within each rating class, affecting the results obtained in downstream data analysis (Tobias

et al., 2018). It is possible that the molecular responses observed are representative of the genetic diversity within the samples, with the HR varying among seedlings within the resistant rating class. This accounts for the variation seen in seedlings rated one, where few showed no visible infection and most showed light chlorotic flecking (Table 2.1). A study conducted by Wang et al. (2018) showed that HR is involved in resistance against anthracnose (*Colletotrichum*) in the tea plant (*Camellia sinensis*). Through transcriptional analysis and histochemistry, the authors found the significant involvement of the HR, where cell walls were reinforced in resistant cultivars as a result of H_2O_2 accumulation. Despite the involvement of HR in resistant cultivars, resistant leaves did not present with physical symptoms of disease or show signs of HR. Despite this, future studies will need to investigate the levels of HR associated compounds, such as H_2O_2, in resistant *E. grandis* to determine if this mechanism is associated with plant resistance at a molecular level, despite the lack of physical evidence of the HR.

Seedlings rated one were considered resistant while seedlings rated five were considered susceptible. This was characterized by either showing few symptoms of infection or large pustules on leaves, shoots and petioles (Table 2.1). Seedlings rated two can also be considered resistant, as the presence of HR is indicative of host resistance against infection. However, the presence of necrosis suggests cell death as the result of disease rather than resistance, as described by Morel and Dangl (1997). Additionally, we observed in some instances that seedlings rated two developed pustules, despite the presence of an HR. For this reason, despite an HR observed in seedlings rated two, we only selected seedlings rated one and five for RNA-seq analysis to investigate the transcriptional responses of the two extremes of host resistance and susceptibility. This is a criteria also selected by Hsieh et al. (2018) when investigating the responses of *M. quinquenervia* upon *A. psidii* infection.

2.5.2 Improper timing and coordination may lead to host susceptibility

Gene ontology biological processes functional characterisation of DEGs in both the R- and S-interactions over time revealed interesting patterns of responses against *A. psidii*. In both interactions, there was significant up-regulation of defence-related terms over time, with few or no down-regulation observed. These responses appeared to begin earlier in the S-interaction, with significant over-representation of defence-related terms observed at 12-hpi. These included responses associated with SA biosynthesis and signalling, hydrogen peroxide metabolism and MAPK cascades (Figure 2.3). A possible explanation for the earlier increased expression of genes may be due to a lack of effective *preformed* defences. A study by Hsieh et al. (2018) suggests that resistance in some individuals of *M. quinquenervia* against myrtle rust may be influenced by preformed defences such as physical barriers

and chemical constituents. The R-interaction may have effective preformed defences that prevent appressorium formation at 12-hpi, while these barriers may be absent more susceptible hosts. Additionally, Xavier et al. (2001) reported that primary hyphae were observed at 12-hpi in susceptible *E. grandis* hosts, while only observed in the resistant hosts at 18-hpi. This is further supported by a study conducted by dos Santos et al. (2019) in which susceptibility in *E. grandis* and *E. phaeotricha* was greatly influenced by the chemical composition of the cuticle waxes. Susceptible hosts lack effective preformed foliar compounds to prevent fungal penetration and as a result, respond to the presence of the pathogen earlier than in resistant hosts.

Despite this observation, the R-interaction shows a rapid increase in responses over 1- and 2-dpi, while similar responses are muted or absent in the S-interaction. Our study suggests that the S-interaction can induce a response against *A. psidii*, as evident by the changes in the transcriptome upon infection. However, it cannot implement adequate and sustained mechanisms to combat disease shown by the absence of critical defence responses by 2-dpi. A recent study showed that susceptible genotypes of *S. luehmannii* had no coordinated systemic response to *A. psidii*, with low host transcript numbers detected (Tobias et al., 2018). This is further supported by Santos et al. (2020), in which susceptible *E. grandis* genotypes were able to activate defence responses after 1-dpi with *A. psidii*, but the responses did not result in successful defence due to a lack of effective signal transduction. This suggests that susceptible *E. grandis* hosts and similarly of other Myrtaceae species, lack coordination in regulation of defence-related genes, which may contribute to disease susceptibility. A previous study on the interaction between *A. psidii* and *M. quinquenervia* showed susceptible plants had various up-regulated defence-related responses at 5-dpi (Hsieh et al., 2018). This is similar to the results obtained in this study, suggesting that Myrtaceae species are able to deploy defences against *A. psidii*, but delays in these responses may contribute to susceptibility. Over-compensation later in the interaction at 5-dpi may be too late to prevent spread of disease.

Interesting KEGG over-represented terms included “photosynthesis – antenna proteins” up-regulated in the R-interaction at 12-hpi, with this term absent in the S-interaction over the time series. Photosynthetic processes are known to play roles in plant defence against pathogenic invasion. Santos et al. (2020) found the constitutive involvement of this pathway in the defence response in resistant *E. grandis* genotypes. Furthermore, “amino sugar and nucleotide sugar metabolism” was unique to the R-interaction at 2-dpi. Sugar signalling has been implicated in disease resistance, with high levels of sugars in plants contributing to defence responses. Increased plant sugars contribute to resistance by inducing expression of *PR* genes, interacting with the plant phytohormone biosynthesis and signalling pathways (Morkunas and Ratajczak, 2014) and increasing production of ROS (Morkunas et al., 2008).

Exogenous applications of sucrose in *Vitis vinifera* induces the production of secondary metabolites such as phenylpropanoid, which may contribute to disease resistance modulated by sugar signalling (Ferri et al., 2011). The up-regulation of phenylpropanoid biosynthesis identified in this study (Figure 4), as well as identification of this pathway in other studies investigating interactions with myrtle rust, suggests the importance of phenylpropanoids in resistance, with sugar metabolism putatively contributing to the induction of this pathway. Moreover, sugar transport, mediated by the sugar transport protein TaHTP, was found to contribute to resistance against the biotrophic rust pathogen *Puccinia triticina* in wheat (Savadi et al., 2018).

2.5.3 *Eucalyptus grandis* phytohormone responses to *Austropuccinia psidii*

Phytohormones contribute significantly to plant defence responses, with crosstalk existing between them to facilitate defence against pathogenic invasion. In this study, genes associated with phytohormones were extensively enriched in both resistant and susceptible *E. grandis* seedlings upon infection with *A. psidii* (Figure 2.3). These include genes relating to SA, JA, ET, ABA, and auxin. Our results suggest putative involvement of SA biosynthesis and signalling, with downstream responses such as systemic acquired resistance identified (Figure 2.3) in both the resistant and susceptible hosts. Santos et al. (2020) found the up-regulation of SA signalling and responsive genes when comparing mock-inoculated myrtle rust resistant and susceptible *E. grandis* genotypes. These findings suggest the importance of SA in *E. grandis* defence against *A. psidii*, both at a constitutive and induced level. Interestingly, GO analysis revealed the involvement of BR at 2-dpi in the R-interaction, a pathway absent in susceptible hosts.

BRs are polyhydroxylated steroidal phytohormones found in plants that have versatile roles in many plant physiological processes. These processes include those such as cell growth and development, seed germination and response to abiotic stress (Saini et al., 2015; Yu et al., 2018; Kim and Russinova, 2020). In recent years, studies have emerged implicating BRs in resistance to various pathogens, with complex molecular interactions driving host responses against pathogenic invasion (Choudhary et al., 2012). BRs have been shown to confer resistance against biotrophic bacteria (Huang et al., 2014) and exogenous application confers systemic resistance to biotrophic fungi such as *Oidium* sp. and *Magnaporthe grisea* (Nakashita et al., 2003).

Many studies on the roles BR play in modulating immune responses have focused on the leucine-rich repeat receptor-like kinase (LRR-RLK) and BRI1 (BR-insensitive 1)-associated kinase (BAK1). BAK1 is a co-receptor of BRI1 and plays a role in BR signal transduction as well as interacting with pattern recognition receptors (PRR) to induce a PTI response against invading pathogens (Li et al.,

2002; Vert, 2008; De Bruyne et al., 2014). Brassinolide (BL), an active form of BR, binds to BRI1 leading to autophosphorylation and the activation of BAK1 (De Bruyne et al., 2014). This ultimately leads to downstream transcriptional changes associated with BR-signalling. BAK1 has been implicated in disease resistance against fungal pathogens including *Sclerotinia sclerotiorum* and *Verticillium dahliae* (Fradin et al., 2009; Chaparro-Garcia et al., 2011) and the identification of nine DE *BAK1* genes in the present study suggests a putative role of BR and associated receptors in the defence response against *A. psidii*. The higher number of DE *BAK1* genes in response to *A. psidii* in the R-interaction may contribute to disease resistance. Similarly, Santos et al. (2020) found constitutive over expression of *BAK1* when comparing mock-inoculated *A. psidii* resistant and susceptible *E. grandis* at 1-dpi, corroborating the findings of the present study. Additionally, in a transcriptome study between banana and *Fusarium oxysporum* f. sp. *cubense*, up-regulation of *BAK1* was identified in relatively resistant banana plants, potentially implicating this gene in the innate immune response in banana (Li et al., 2013).

In addition to BAK1, the somatic embryogenesis receptor-like protein kinase-1 (SERK1) are implicated in disease resistance in tomato against the foliar fungal pathogen, *Cladosporium fulvum* (Fradin et al., 2011). The SERK protein family, which includes SERK3/BAK1 has been extensively shown to contribute to disease resistance. In this study, five *SERK1* genes were DE in *E. grandis* upon challenge by *A. psidii* (Figure 2.4). The expression of these genes is similar to that of *BAK1*. Further analyses of the genes involved in BR signalling revealed DE of gene BZR1 encoding brassinazole-resistant 1. This is a transcription factor responsible for repressing expression of defence-related genes (Robert-Seilaniantz et al., 2011). Further studies into the role this gene is playing during the interaction of *E. grandis* are required. However, up-regulation of this gene was low or absent over the time course within both interactions. This suggests defence-related responses involved in PTI may not be repressed.

Despite our results suggesting a role of BR signalling in defence against *A. psidii*, studies on the effector AVR2 have shown that increased BR signalling in transgenic potato plants leads to enhanced susceptibility to *Phytophthora infestans*. This effector manipulates the crosstalk that exists between plant growth and immunity to facilitate enhanced pathogen colonization (Turnbull et al., 2017). This highlights the complexities of BR signalling and the roles this phytohormone pathway plays in plant defence. Different pathogens employ different virulence mechanisms to elicit disease in host plants, and this may explain the different outcomes observed in BR signalling between *P. infestans (*oomycete) and *A. psidii* (fungus). Moreover, the lifestyle of the pathogen contributes to the outcome of BR in plant defence. Despite AVR2 being up-regulated during the biotrophic phase in *P. infestans*,

this oomycete is a hemi-biotroph while *A. psidii* is a biotroph and studies have shown that BR increases plant defence in response to biotrophs while decreasing plant defence against hemibiotrophs and necrotrophs (De Vleesschauwer et al., 2012; Turnbull et al., 2017; Yu et al., 2018), this contributes further to the complexities of this phytohormone in plant responses. For this reason, more studies are required to understand how BRs contribute to the responses of *E. grandis* to *A. psidii*.

The identification of BR mediated signalling genes underlying *A. psidii* resistance loci may implicate this pathway in the resistance conferred by these loci. *Ppr3* harbours two genes that are associated with BR signalling and BR receptors, while *Ppr5* harbours four genes that code leucine-rich receptor proteins and protein kinases. Eucgr.G01832 (BRI1-interacting signalling kinase, BSK1) underlies *Ppr3,* while Eucgr.I00302 and Eucgr.I00303 (BRI1) underlies *Ppr5* (Supplementary Table 2.4). BRs associates with BRI1, causing phosphorylation and subsequent dissociation of BRI1 from its associated inhibitors. This cascade event leads to the activation of BAK1 and the phosphorylation of BSK1 (De Bruyne et al., 2014; Yu et al., 2018). This causes activation of downstream BR signalling responses and changes in gene expression. Despite these loci harbouring BR signalling genes, these were not DE in this study. However, extensive polymorphisms may exist within these genes that affect the expression as well as the final protein product, which may contribute to BR signalling and resistance against *A. psidii*. Further studies are required to identify single nucleotide polymorphisms (SNPs) that may exist between resistant and susceptible seedlings to determine how these genes may contribute to disease resistance.

2.5.4 Constitutive and induced expression of candidate resistant genes

Plants recognize pathogen effectors through resistance proteins, most commonly the nucleotide-binding domains and leucine-rich repeats (NLRs) (Jones and Dangl, 2006). Effectors are small secreted proteins (SSPs) that the pathogen deploys to facilitate the manipulation of host responses, allowing the pathogen to acquire host nutrients and reduce plant defence (Fudal et al., 2018). The recognition of these pathogenic effector proteins leads to effector-triggered immunity (ETI), resulting in oxidative burst of ROS as well as hypersensitive responses (HR) (Jones and Dangl 2006) . These responses lead to extensive modifications in defence-related gene expression and prevention of pathogen spread from the site of infection (Dodds and Rathjen, 2010).

In *Eucalyptus,* numerous *R*-gene loci conferring resistance to *A. psidii* have been discovered (Junghans et al., 2003a; Butler et al., 2016). *Ppr1* confers resistance to myrtle rust in *E. grandis* while *Ppr2-5* has been shown to confer resistance to myrtle rust in *E. globulus,* a close relative of *E. grandis* (Butler et al., 2016). Little is known about the changes in expression over time of genes associated with these

loci and how they contribute to disease resistance. To further investigate the genes underlying these loci, we identified various DEGs in the interaction between *A. psidii* and *E. grandis,* many of which were *R*-genes (Figure 2.6 and Figure 2.7A-I, Supplementary Table 2.3). In general, there were more significantly DE *R*-genes in the R-interaction underlying these loci than in the S-interaction. Additionally, the R-interaction showed significant DE predominately between 1- and 2-dpi, while significant expression was only observed in the S-interaction during later stage infection. A recent study by Santos et al. (2020) identified two significantly DE *R*-genes underlying *Ppr1* when investigating mock-inoculated resistant and susceptible *E. grandis* as well as susceptible infected and control *E. grandis*, namely Eucgr.C02650 and Eucgr.C02749. Eucgr.C02650 was down-regulated in both, while Eucgr.C02749 was up-regulated in both. In our study, we found significant DE of Eucgr.C02650 when investigating resistant, infected relative to control as well as investigations between resistant and susceptible controls.

Ppr2 and *Ppr3* determine if the plant shows disease symptoms (Butler et al., 2016). In the R-interaction, these loci showed significant up-regulation at 1- and 2-dpi. In contrast, while the expression patterns are similar, the S-interaction showed fewer significant DE of these *R*-genes. This may contribute to the disease symptoms observed in susceptible seedlings. *Ppr4* and *Ppr5* determine if the plant exhibits a hypersensitive response (Butler et al., 2016). Both hosts showed down-regulation of these genes in *Ppr4,* suggesting the pathogen is potentially manipulating the HR that are controlled by these *R*-genes (Figure 2.6). Despite this, there was significant expression of some of these *R*-genes in both interactions at *Ppr4* showing high TPM values. Similarly, at *Ppr5,* the R-interaction shows more DE than in the S-interaction, with significant up-regulation in the R-interaction at 2-dpi. This suggests an involvement of these *R*-genes in the HR observed in the resistant hosts. This observation correlates with those made in the GO responses in which there is a delay in HR in the S-interaction.

It is important for plant defence responses to be suppressed in the absence of plant pathogens, as constitutive expression of defence-related compounds may be detrimental to plant growth and development (Marone et al., 2013). However, constitutive expression of defence-related genes, including *R*-genes, has been shown to contribute to disease resistance against pathogens. For example, in *Arabidopsis ADR1* codes a specific CC-NB-LRR resistance gene. Mutations in this gene led to the constitutive expression of this *R*-gene, conferring broad-spectrum disease resistance against biotrophic pathogens and drought tolerance (Grant et al., 2003; Chini et al., 2004). Furthermore, constitutive expression of a TIR-NB-ARC-LRR disease resistance protein conferred resistance to phytopathogenic fungi and bacteria (Wen et al., 2017). Our results suggest that the constitutive expression of *R*-genes underlying resistance QTLs may putatively contribute to disease resistance. These results corroborate

those from a recent study investigating the transcriptome of *E. grandis*, which found constitutive expression of genes encoding R proteins, such as Eucgr.C02650 underlying *Ppr1,* that was found to be significantly constitutively DE in the present study. The authors suggest this constitutive expression of defence-related genes potentially contributes to disease resistance observed against *A. psidii* (Santos et al., 2020).

The identification of DE *R*-genes when investigating the *E. grandis* transcriptomes, both at the induced and constitutive levels highlights candidate genes putatively involved in the defence responses and further investigations into the molecular differences that exist within these genes between genotypes is required to elucidate their roles.

2.6 Conclusions

This study investigated the molecular interactions of *E. grandis* in response to *A. psidii* to investigate mechanisms underlying host resistance. We identified pathways and genes potentially involved in the interaction. This includes brassinosteroid signalling, a pathway not previously reported in the *A. psidii*-host interactions and various *R*-genes that have different expression patterns, both at the constitutive and induced levels. Moreover, our findings suggest that resistant and susceptible hosts share similar responses, but timing and intensity determines disease severity. Due to the bioinformatic nature of RNA-seq analysis, further functional studies are required to characterize the observed responses to validate the results in the present study. This includes studies that investigate the brassinosteroid levels between resistant and susceptible seedlings to confirm the involvement of this pathway in host resistance. Moreover, investigations into the identified resistance genes are required to elucidate the roles they play during the interaction. Future studies should also aim to include investigations of the pathogen responses to identify mechanisms *A. psidii* is employing to manipulate susceptible hosts, as it will provide insight into novel targets of pathogen control, facilitated by the use of the *A. psidii* reference genome (Tobias et al., 2021). In conclusion, the results from this study will contribute to improved selection and breeding of *E. grandis* by manipulating pathways and genes tagged as important contributors to resistance.

2.7 References

Butler, J.B., Freeman, J.S., Vaillancourt, R.E., Potts, B.M., Glen, M., Lee, D.J., et al. (2016). Evidence for different QTL underlying the immune and hypersensitive responses of *Eucalyptus globulus* to the rust pathogen *Puccinia psidii*. *Tree Genetics & Genomes* 12**,** 39. doi: 10.1007/s11295-016-0987-x.

Carnegie, A.J., Kathuria, A., Pegg, G.S., Entwistle, P., Nagel, M., and Giblin, F.R. (2016). Impact of the invasive rust Puccinia psidii (myrtle rust) on native Myrtaceae in natural ecosystems in Australia. *Biological Invasions* 18**,** 127-144.

Carnegie, A.J., Lidbetter, J.R., Walker, J., Horwood, M.A., Tesoriero, L., Glen, M., et al. (2010). *Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia. *Australasian Plant Pathology* 39**,** 463-466. doi: 10.1071/AP10102.

Carnegie, A.J., and Pegg, G.S. (2018). Lessons from the incursion of myrtle rust in Australia. *Annual Review of Phytopathology* 56**,** 457-478. doi: 10.1146/annurev-phyto-080516-035256.

Chaparro-Garcia, A., Wilkinson, R.C., Gimenez-Ibanez, S., Findlay, K., Coffey, M.D., Zipfel, C., et al. (2011). The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen *Phytophthora infestans* in *Nicotiana benthamiana*. *PloS One* 6**,** e16608. doi: 10.1371/journal.pone.0016608.

Chini, A., Grant, J.J., Seki, M., Shinozaki, K., and Loake, G.J. (2004). Drought tolerance established by enhanced expression of the *CC–NBS–LRR* gene, *ADR1*, requires salicylic acid, EDS1 and ABI1. *The Plant Journal* 38**,** 810-822. doi: 10.1111/j.1365-313X.2004.02086.x.

Choudhary, S.P., Yu, J.-Q., Yamaguchi-Shinozaki, K., Shinozaki, K., and Tran, L.-S.P. (2012). Benefits of brassinosteroid crosstalk. *Trends in Plant Science* 17**,** 594-605. doi: 10.1016/j.tplants.2012.05.012.

Coutinho, T.A., Wingfield, M.J., Alfenas, A.C., and Crous, P.W. (1998). *Eucalyptus* Rust: A disease with the potential for serious international implications. *Plant Disease* 82**,** 819-825. doi: 10.1094/PDIS.1998.82.7.819.

De Bruyne, L., Höfte, M., and De Vleesschauwer, D. (2014). Connecting growth and defense: The emerging roles of brassinosteroids and gibberellins in plant innate immunity. *Molecular Plant* 7**,** 943-959. doi: 10.1093/mp/ssu050.

De Vleesschauwer, D., Van Buyten, E., Satoh, K., Balidion, J., Mauleon, R., Choi, I.R., et al. (2012). Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice. *Plant Physiology* 158**,** 1833-1846. doi: 10.1104/pp.112.193672.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics* 11**,** 539-548. doi: 10.1038/nrg2812.

dos Santos, I.B., Lopes, M.D.S., Bini, A.P., Tschoeke, B.a.P., Verssani, B.a.W., Figueredo, E.F., et al. (2019). The *Eucalyptus* cuticular waxes contribute in preformed defense against *Austropuccinia psidii*. *Frontiers in Plant Science* 9**,** 1978. doi: 10.3389/fpls.2018.01978.

Du Plessis, E., Granados, G.M., Barnes, I., Ho, W.H., Alexander, B.J.R., Roux, J., et al. (2019). The pandemic strain of *Austropuccinia psidii* causes myrtle rust in New Zealand and Singapore. *Australasian Plant Pathology* 48**,** 253-256. doi: 10.1007/s13313-019-0624-x.

Fensham, R.J., Carnegie, A.J., Laffineur, B., Makinson, R.O., Pegg, G.S., and Wills, J. (2020). Imminent extinction of Australian Myrtaceae by fungal disease. *Trends in Ecology & Evolution* 35**,** 7. doi: 10.1016/j.tree.2020.03.012.

Ferri, M., Righetti, L., and Tassoni, A. (2011). Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. *Journal of Plant Physiology* 168**,** 189-195. doi: 10.1016/j.jplph.2010.06.027.

Fradin, E.F., Abd-El-Haliem, A., Masini, L., Van Den Berg, G.C.M., Joosten, M.H.a.J., and Thomma, B.P.H.J. (2011). Interfamily transfer of tomato *Ve1* mediates *Verticillium* resistance in Arabidopsis. *Plant Physiology* 156**,** 2255-2265. doi: 10.1104/pp.111.180067.

Fradin, E.F., Zhang, Z., Juarez Ayala, J.C., Castroverde, C.D.M., Nazar, R.N., Robb, J., et al. (2009). Genetic dissection of *Verticillium* wilt resistance mediated by tomato *Ve1*. *Plant Physiology* 150**,** 320-332. doi: 10.1104/pp.109.136762.

Fudal, I., Balesdent, M.-H., and Rouxel, T. (2018). Effector biology in fungal pathogens of nonmodel crop plants. *Trends in Plant Science* 23**,** 753-755. doi: 10.1016/j.tplants.2018.07.002.

Giblin, F. (2013). Myrtle rust report: New Caledonia. *University of the Sunshine Coast, Maroochydore, Queensland*.

Glen, M., Alfenas, A.C., Zauza, E.a.V., Wingfield, M.J., and Mohammed, C. (2007). *Puccinia psidii*: A threat to the Australian environment and economy - A review. *Australasian Plant Pathology* 36**,** 1-16. doi: 10.1071/AP06088.

Goecks, J., Nekrutenko, A., Taylor, J., and Team, G. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome biology* 11**,** R86. doi: 10.1186/gb-2010-11-8-r86.

Graça, R.N., Aun, C.P., Guimarães, L.M.S., Rodrigues, B.V.A., Zauza, E.a.V., and Alfenas, A.C. (2011). A new race of *Puccinia psidii* defeats rust resistance in eucalypt. *Australasian Plant Pathology* 40**,** 442-447. doi: 10.1007/s13313-011-0056-8.

Grant, J.J., Chini, A., Basu, D., and Loake, G.J. (2003). Targeted activation tagging of the Arabidopsis *NBS-LRR* gene, *ADR1*, conveys resistance to virulent pathogens. *Molecular Plant-Microbe Interactions* 16**,** 669-680. doi: 10.1094/MPMI.2003.16.8.669.

Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32**,** 2847-2849. doi: 10.1093/bioinformatics/btw313.

Hahn, M., and Mendgen, K. (2001). Signal and nutrient exchange at biotrophic plant-fungus interfaces. *Current Opinion in Plant Biology* 4**,** 322-327. doi: 10.1016/S1369-5266(00)00180-1.

Hsieh, J.-F., Chuah, A., Patel, H.R., Sandhu, K.S., Foley, W.J., and KüLheim, C. (2018). Transcriptome profiling of *Melaleuca quinquenervia* challenged by myrtle rust reveals differences in defense responses among resistant individuals. *Phytopathology* 108**,** 495-509. doi: 10.1094/PHYTO-09-17-0307-R.

Huang, P.-Y., Yeh, Y.-H., Liu, A.-C., Cheng, C.-P., and Zimmerli, L. (2014). The Arabidopsis LecRK-VI.2 associates with the pattern-recognition receptor FLS2 and primes *Nicotiana benthamiana* pattern-triggered immunity. *The Plant Journal* 79**,** 243-255. doi: 10.1111/tpj.12557.

Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. *Nature* 444**,** 323-329. doi: 10.1038/nature05286.

Junghans, D.T., Alfenas, A.C., Brommonschenkel, S.H., Oda, S., Mello, E.J., and Grattapaglia, D. (2003a). Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. *Theoretical and Applied Genetics* 108**,** 175-180. doi: 10.1007/s00122-003-1415-9.

Junghans, D.T., Alfenas, A.C., and Maffia, L.A. (2003b). Escala de notas para quantificação da ferrugem em Eucalyptus. *Fitopatologia Brasileira* 28**,** 184-188.

Kawanishi, T., Uematsu, S., Kakishima, M., Kagiwada, S., Hamamoto, H., Horie, H., et al. (2009). First report of rust disease on ohia and the causal fungus, *Puccinia psidii*, in Japan. *Journal of General Plant Pathology* 75**,** 428-431. doi: 10.1007/s10327-009-0202-0.

Kim, E.-J., and Russinova, E. (2020). Brassinosteroid signalling. *Current Biology* 30**,** R294-R298. doi: 10.1016/j.cub.2020.02.011.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9**,** 357-359. doi: 10.1038/nmeth.1923.

Li, C.Q., Shao, J.F., Wang, Y.J., Li, W.B., Guo, D.J., Yan, B., et al. (2013). Analysis of banana transcriptome and global gene expression profiles in banana roots in response to infection by race 1 and tropical race 4 of *Fusarium oxysporum* f. sp *cubense*. *BMC Genomics* 14**,** 16. doi: 10.1186/1471-2164-14-851.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110**,** 213-222. doi: 10.1016/s0092-8674(02)00812-7.

Liao, Y., Smyth, G.K., and Shi, W. (2013). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30**,** 923-930. doi: 10.1093/bioinformatics/btt656.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15**,** 550. doi: 10.1186/s13059-014-0550-8.

Mamani, E.M., Bueno, N.W., Faria, D.A., Guimarães, L.M., Lau, D., Alfenas, A.C., et al. (2010). Positioning of the major locus for *Puccinia psidii* rust resistance (*Ppr1*) on the *Eucalyptus* reference map and its validation across unrelated pedigrees. *Tree Genetics & Genomes* 6**,** 953-962. doi: 10.1007/s11295-010-0304-z.

Marlatt, R.B., and Kimbrough, J.W. (1979). *Puccinia psidii* on *Pimenta dioica* in south Florida. *Plant Disease Reporter* 63**,** 510-512.

Marone, D., Russo, M.A., Laido, G., De Leonardis, A.M., and Mastrangelo, A.M. (2013). Plant nucleotide binding site-leucine-rich repeat (NBS-LRR) genes: Active guardians in host defense responses. *International Journal of Molecular Sciences* 14**,** 7302-7326. doi: 10.3390/ijms14047302.

Mctaggart, A.R., Roux, J., Granados, G.M., Gafur, A., Tarrigan, M., Santhakumar, P., et al. (2016). Rust (*Puccinia psidii*) recorded in Indonesia poses a threat to forests and forestry in South-East Asia. *Australasian Plant Pathology* 45**,** 83-89. doi: 10.1007/s13313-015-0386-z.

Morel, J.-B., and Dangl, J.L. (1997). The hypersensitive response and the induction of cell death in plants. *Cell Death & Differentiation* 4**,** 671-683. doi: 10.1038/sj.cdd.4400309.

Morkunas, I., Bednarski, W., and Kopyra, M. (2008). Defense strategies of pea embryo axes with different levels of sucrose to *Fusarium oxysporum* and *Ascochyta pisi*. *Physiological and Molecular Plant Pathology* 72**,** 167-178. doi: 10.1016/j.pmpp.2008.09.003.

Morkunas, I., and Ratajczak, L. (2014). The role of sugar signaling in plant defense responses against fungal pathogens. *Acta Physiologiae Plantarum* 36**,** 1607-1619. doi: 10.1007/s11738-014-1559-z.

Myburg, A.A., Grattapaglia, D., Tuskan, G.A., Hellsten, U., Hayes, R.D., Grimwood, J., et al. (2014). The genome of *Eucalyptus grandis*. *Nature* 510**,** 356. doi: 10.1038/nature13308.

Naidoo, S., KüLheim, C., Zwart, L., Mangwanda, R., Oates, C.N., Visser, E.A., et al. (2014). Uncovering the defence responses of *Eucalyptus* to pests and pathogens in the genomics age. *Tree Physiology* 34**,** 931-943. doi: 10.1093/treephys/tpu075.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., et al. (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *The Plant Journal* 33**,** 887-898. doi: 10.1046/j.1365-313x.2003.01675.x.

Pegg, G.S., Brawner, J., and Lee, D. (2014). Screening *Corymbia* populations for resistance to *Puccinia psidii*. *Plant Pathology* 63**,** 425-436. doi: 10.1111/ppa.12097.

Pegg, G.S., Entwistle, P., Giblin, F.R., and Carnegie, A.J. (2020). Fire and rust – the impact of *Austropuccinia psidii* (myrtle rust) on regeneration of Myrtaceae in coastal heath following wildfire. *Southern Forests: A Journal of Forest Science* 82**,** 280-291. doi: 10.2989/20702620.2020.1819154.

Pegg, G.S., Taylor, T., Entwistle, P., Guymer, G., Giblin, F., and Carnegie, A. (2017). Impact of Austropuccinia psidii (myrtle rust) on Myrtaceae-rich wet sclerophyll forests in south east Queensland. *PLoS One* 12. doi: 10.1371/journal.pone.0188058.

R Core Team (2018). "R: A language and environment for statistical computing", in: *R Foundation for Statistical Computing, Vienna, Austria. URL* https://www.R-project.org/.).

Rayachhetry, M.B., Elliott, M.L., and Van, T.K. (1997). Natural epiphytotic of the rust *Puccinia psidii* on *Melaleuca quinquenervia* in Florida. *Plant Disease* 81**,** 831. doi: 10.1094/pdis.1997.81.7.831a.

Robert-Seilaniantz, A., Grant, M., and Jones, J.D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology* 49**,** 317-343. doi: 10.1146/annurev-phyto-073009-114447.

Roux, J., Greyling, I., Coutinho, T.A., Verleur, M., and Wingfield, M.J. (2013). The Myrtle rust pathogen, *Puccinia psidii*, discovered in Africa. *IMA fungus* 4**,** 155-159. doi: 10.5598/imafungus.2013.04.01.14.

Saini, S., Sharma, I., and Pati, P.K. (2015). Versatile roles of brassinosteroid in plants in the context of its homoeostasis, signaling and crosstalks. *Frontiers in Plant Science* 6**,** 950. doi: 10.3389/fpls.2015.00950.

Santos, S.A., Vidigal, P.M.P., GuimarãEs, L.M.S., Mafia, R.G., Templeton, M.D., and Alfenas, A.C. (2020). Transcriptome analysis of *Eucalyptus grandis* genotypes reveals constitutive overexpression of genes related to rust (*Austropuccinia psidii*) resistance. *Plant Molecular Biology*. doi: 10.1007/s11103-020-01030-x.

Savadi, S., Prasad, P., Bhardwaj, S.C., Kashyap, P.L., Gangwar, O.P., Khan, H., et al. (2018). Temporal transcriptional changes in SAR and sugar transport-related genes during wheat and leaf rust pathogen interactions. *Journal of Plant Growth Regulation* 37**,** 826-839. doi: 10.1007/s00344-017-9777-4.

Sekiya, A., Marques, F.G., Leite, T.F., Cataldi, T.R., De Moraes, F.E., Pinheiro, A.L.M., et al. (2021). Network analysis combining proteomics and metabolomics reveals new insights into early responses of *Eucalyptus grandis* during rust infection. *Frontiers in Plant Science* 11. doi: 10.3389/fpls.2020.604849.

Soewarto, J., Giblin, F., and Carnegie, A.J. (2019). *Austropuccinia psidii (myrtle rust) global host list. Version 4* [Online]. Canberra, ACT: Australian Network for Plant Conservation. http://www.anpc.asn.au/myrtle-rust. Accessed 20 November 2020. Available: http://www.anpc.asn.au/myrtle-rust [Accessed 20 November 2020].

Thumma, B., Pegg, G.S., Warburton, P., Brawner, J.T., Macdonell, P., Yang, X., et al. (2013). Molecular tagging of rust resistance genes in eucalypts. 1-15.

Tobias, P.A., Guest, D.I., Külheim, C., and Park, R.F. (2018). De novo transcriptome study identifies candidate genes involved in resistance to *Austropuccinia psidii* (myrtle rust) in *Syzygium luehmannii* (riberry). *Phytopathology* 108**,** 627-640. doi: 10.1094/PHYTO-09-17-0298-R.

Tobias, P.A., Schwessinger, B., Deng, C.H., Wu, C., Dong, C., Sperschneider, J., et al. (2021). *Austropuccinia psidii,* causing myrtle rust, has a gigabase-sized genome shaped by transposable elements. *G3 Genes|Genomes|Genetics* 11**,** jkaa015. doi: 10.1093/g3journal/jkaa015.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols* 7**,** 562. doi: 10.1038/nprot.2012.016.

Turnbull, D., Yang, L., Naqvi, S., Breen, S., Welsh, L., Stephens, J., et al. (2017). RXLR effector AVR2 up-regulates a brassinosteroid-responsive bHLH transcription factor to suppress immunity. *Plant Physiology* 174**,** 356-369. doi: 10.1104/pp.16.01804.

Uchida, J., Zhong, S., and Killgore, E. (2006). First report of a rust disease on Ohia caused by *Puccinia psidii* in Hawaii. *Plant Disease* 90**,** 524. doi: 10.1094/pd-90-0524c.

Vert, G. (2008). Plant signaling: brassinosteroids, immunity and effectors are BAK! *Current Biology* 18**,** R963-R965. doi: 10.1016/j.cub.2008.09.006.

Wang, Y., Hao, X., Lu, Q., Wang, L., Qian, W., Li, N., et al. (2018). Transcriptional analysis and histochemistry reveal that hypersensitive cell death and H2O2 have crucial roles in the resistance of tea plant (*Camellia sinensis* (L.) O. Kuntze) to anthracnose. *Hortic. Res.-England* 5**,** 18. doi: 10.1038/s41438-018-0025-2.

Wen, Z., Yao, L., Singer, S.D., Muhammad, H., Li, Z., and Wang, X. (2017). Constitutive heterologous overexpression of a TIR-NB-ARC-LRR gene encoding a putative disease resistance protein from wild Chinese *Vitis pseudoreticulata* in Arabidopsis and tobacco enhances resistance to phytopathogenic fungi and bacteria. *Plant Physiology and Biochemistry* 112**,** 346-361. doi: 10.1016/j.plaphy.2017.01.017.

Xavier, A.A., Alfenas, A.C., Matsuoka, K., and Hodges, C.S. (2001). Infection of resistant and susceptible *Eucalyptus grandis* genotypes by urediniospores of *Puccinia psidii*. *Australasian Plant Pathology* 30**,** 277-281. doi: 10.1071/ap01038.

Xavier, A.A., Da Silva, A.C., Da Silva Guimarães, L.M., Matsuoka, K., Hodges, C.S., and Alfenas, A.C. (2015). Infection process of *Puccinia psidii* in *Eucalyptus grandis* leaves of different ages. *Tropical Plant Pathology* 40**,** 318-325. doi: 10.1007/s40858-015-0043-7.

Yu, M.-H., Zhao, Z.-Z., and He, J.-X. (2018). Brassinosteroid signaling in plant-microbe interactions. *International Journal of Molecular Sciences* 19**,** 4091. doi: 10.3390/ijms19124091.

Zeng, Y., and Yang, T. (2002). RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter* 20**,** 417-417. doi: 10.1007/BF02772130.

Zhuang, J., and Wei, S. (2011). Additional materials for the rust flora of Hainan Province, China. *Mycosystema* 30**,** 853-860.

2.8 Tables and figures

Table 2.1. Progression of disease symptoms observed on *Eucalyptus grandis* in response to *Austropuccinia psidii.*

Ratings[a]	Pustule size	Disease symptoms
1	None present	No symptoms evident or presence of flecking (chlorotic/clear)
2	None present	Presence of a hypersensitive response with flecking or necrosis
3	<0.8 mm	Small pustules, with one or two uredinia
4	0.8-1.6 mm	Medium-sized pustules, with approximately 12 uredinia
5	>1.6 mm	Large pustules, with more than 20 uredinia on leaves, petioles and/or shoots

a Based on parameters defined by Junghans et al. (2003b).

Table 2.2. *Austropuccinia psidii* rust resistance QTL and their physical positions on linkage groups.

QTL	LG	Physical position (bp) of flanking markers		Reference
Ppr1	3	51,630,679	61,298,022	Junghans et al. (2003a)
Ppr2	3	28,711,882	42,683,460	Butler et al. (2016)
Ppr3	7	31,587,467	45,879,882	Butler et al. (2016)
Ppr4	6	9,239,564	17,425,979	Butler et al. (2016)
Ppr5	9	1,718,324	23,366,994	Butler et al. (2016)

QTL quantitative trait loci; *LG* linkage group; *bp* base pairs; *Ppr- Puccinia psidii resistance* loci

Table 2.3. The total number of genes identified underlying known resistance loci, showing the number of total and differentially expressed R-genes in both resistant and susceptible hosts.

Loci	Total genes	DEGs[a]			Total *R*-genes	DE *R*-genes[a]		
		R	S	S-C vs. R-C		R	S	S-C vs. R-C
Ppr1	382	45	23	4	34	8	2	2
Ppr2	459	45	34	7	35	7	5	4
Ppr3	752	86	56	4	25	6	1	0
Ppr4	506	93	45	3	54	9	7	2
Ppr5	941	134	74	3	15	6	4	1

R = resistant; S = susceptible; R-C = resistant, control; S-C = susceptible, control.

[a] Genes were considered differentially expressed with a $\log_2$ (fold change) < -1.00 and > 1.00 in at least one time point.

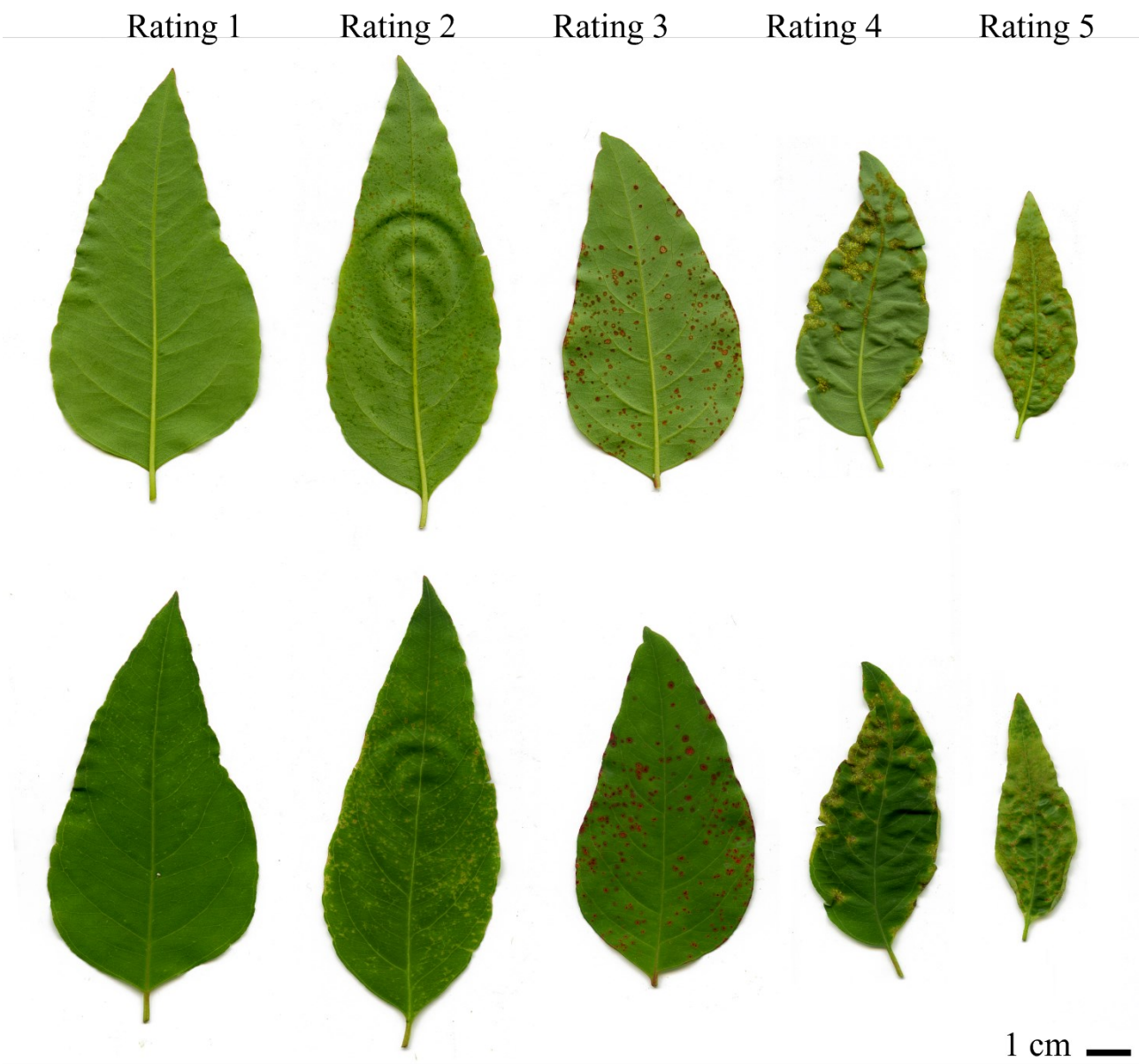

Figure 2.1. Disease progression of *Eucalyptus grandis* in response to *Austropuccinia psidii*. The top leaves show the adaxial while the bottom shows the abaxial view of the leaves. Rating one was considered resistant while ratings two to five were considered susceptible to *A. psidii.* Rating one showed slight chlorotic flecking, progressing in rating two and three where the flecking became necrotic and uredinia pustules began to form. Uredinia pustules became more apparent in rating four and severe pustules began forming by rating five. Rating one and rating five were selected for further analysis.

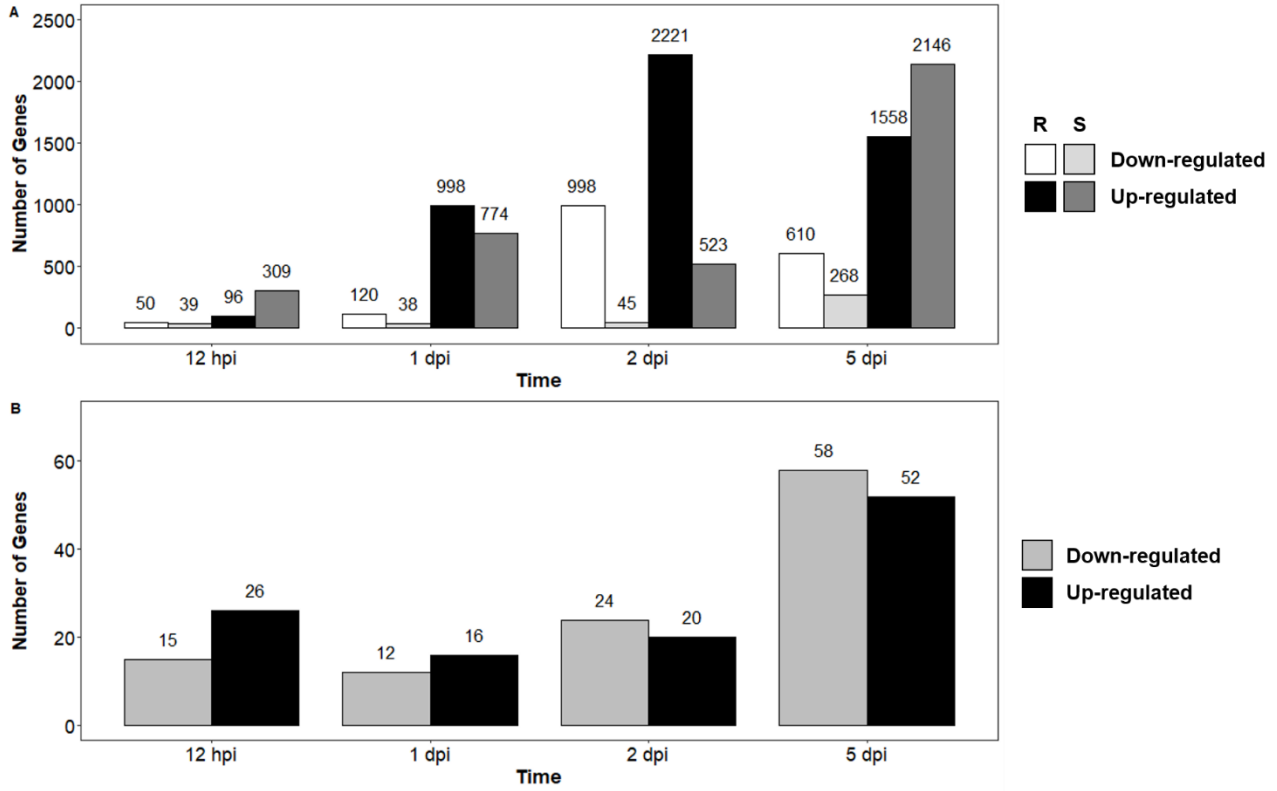

Figure 2.2. Number of differentially expressed genes (DEGs) identified through RNA-seq analysis over the time series in **(A)** resistant (R-interaction) and susceptible (S-interaction) and **(B)** susceptible, control compared to resistant, control (S-C vs. R-C) where grey bars represent genes greater expressed in the S-interaction and black bars represent genes greater expressed in the R-interaction. Genes considered down-regulation with a $\log_2$ (fold change) < -1.00 and up-regulated with $\log_2$ fold change > 1.00.

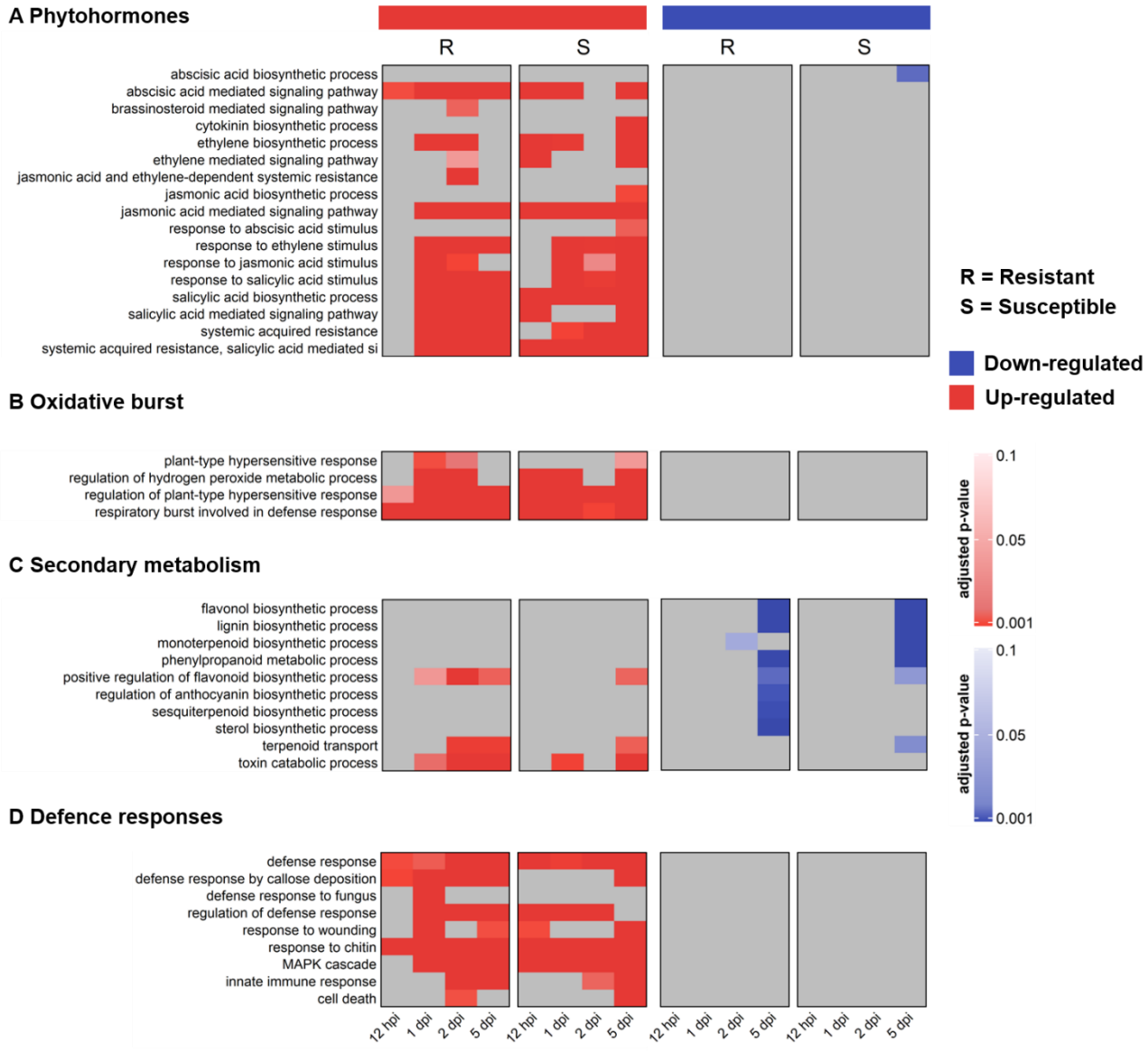

Figure 2.3. Gene ontology (GO) biological processes (BP) functional characterisation of significantly differentially expressed genes (DEGs) in the R- and S-interactions, showing selected defence-related terms over time. Red bars represent up-regulated, and blue represents down-regulated GO terms. Colour bars indicate significance with an adjusted p-value ≤ 0.05.

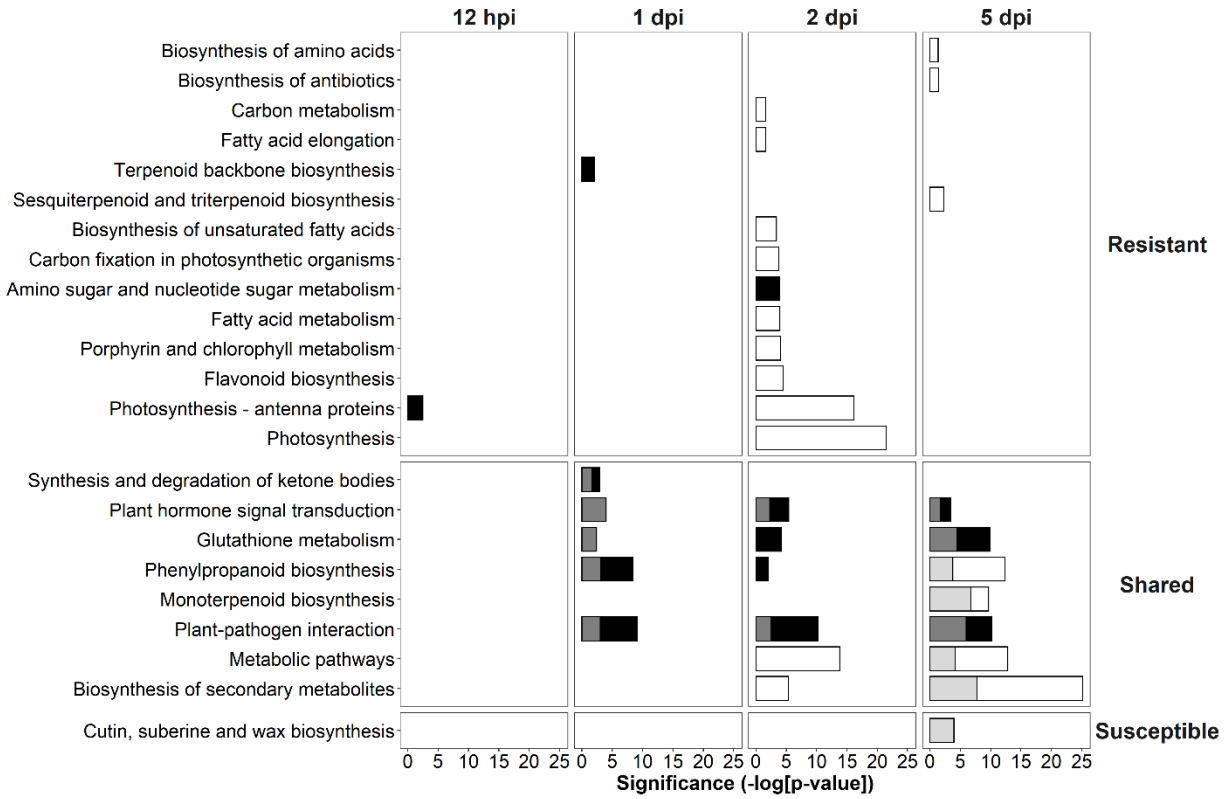

Figure 2.4. Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis of significantly differentially expressed genes (DEGs) in the R- and S-interactions over time showing the significance of each over-represented term. White bars indicate down-regulated resistant, black bars indicate up-regulated resistant, light grey bars indicate down-regulated susceptible and dark grey bars indicate up-regulated susceptible. KEGG terms were separated to those unique to the R-interaction, shared in both the R- and S-interactions and unique to the S-interaction.

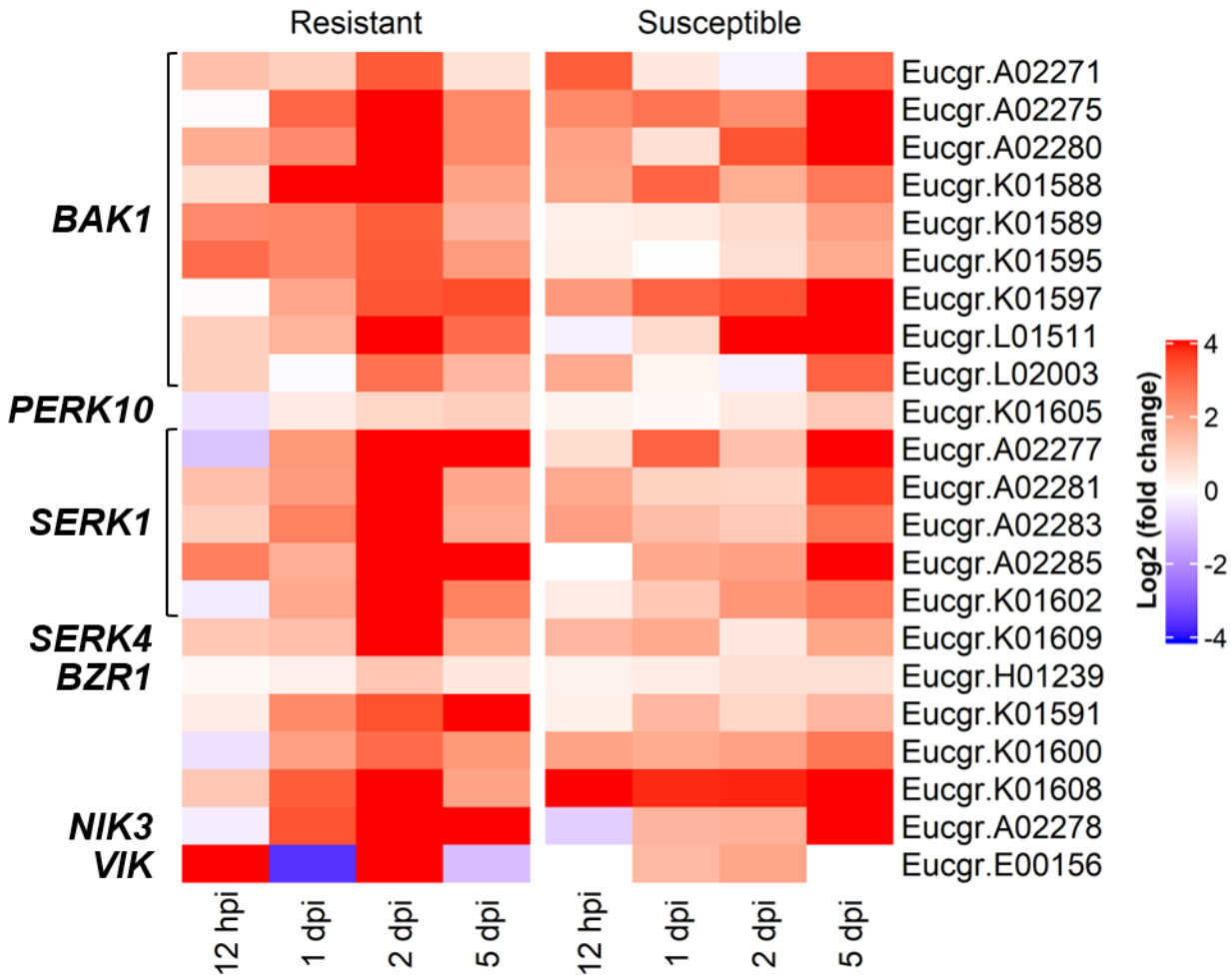

Figure 2.5. **The brassinosteroid mediated signalling pathway** associated genes showing differentially expressed genes (DEGs) in both the R- and S-interactions over time, where red and blue indicate up- and down-regulation, respectively (log_2 (fold change)). These genes were identified in GO BP enrichment.

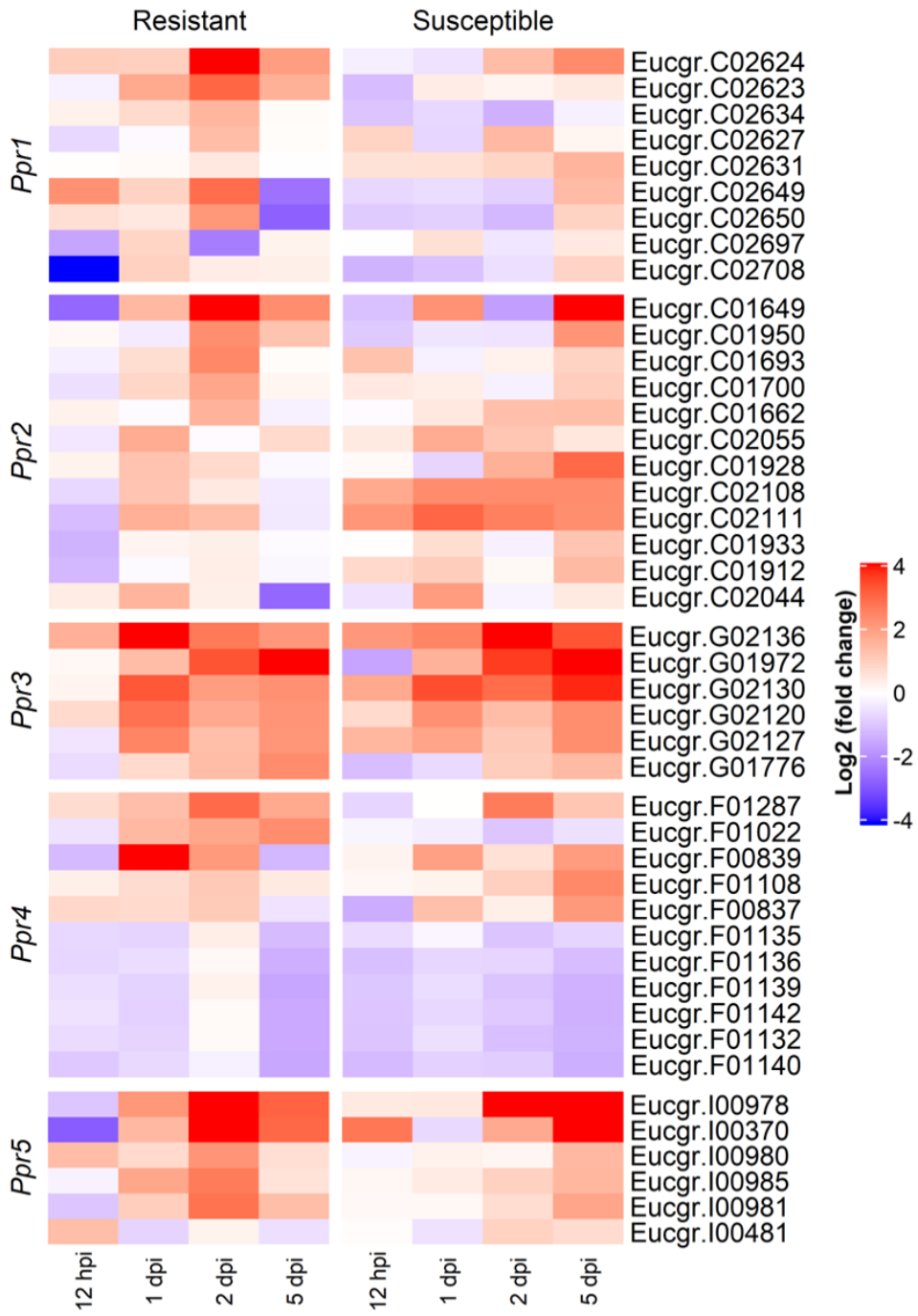

Figure 2.6. Heatmap showing the expression profiles of differentially expressed R (resistance) genes underlying the *Austropuccinia psidii* resistance loci in the R- and S-interactions over time, where red and blue represent up- and down-regulated genes, respectively. Heatmap shows $\log_2$ (fold change) with $p < 0.001$ representing significant DE.

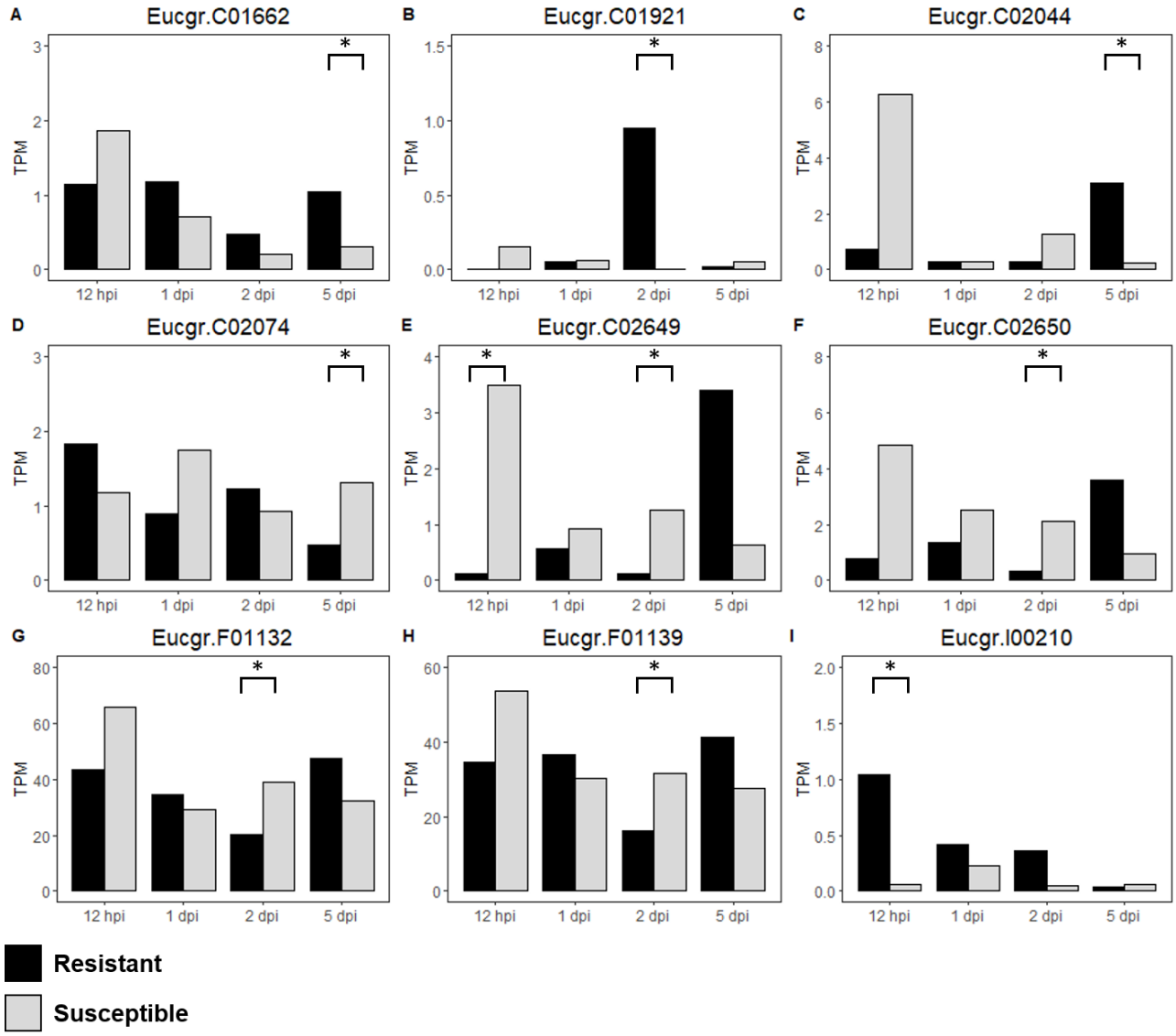

Figure 2.7. Differentially expressed (DE) R-genes underlying the *Austropuccinia psidii* resistance loci in the susceptible, control compared to resistant, control seedlings over the time series, representing TPM values of DE R-genes in all biological replicates in the controls of the R- and S-interactions i.e. constitutive expression of the R-genes are being compared, where black represents resistant and light grey represents susceptible seedlings and asterixis represent significant DE.

Chapter 3

Research Chapter

The *in planta* gene expression of *Austropuccinia psidii* in resistant and susceptible *Eucalyptus grandis*

Shae Swanepoel [1], Caryn N. Oates [1], Louise S. Shuey [2], Geoff Pegg [2], Sanushka Naidoo [1]

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

[2] Department of Agriculture and Fisheries, Queensland Government, Australia

This chapter has been prepared in the format of a manuscript for a peer-reviewed research journal. Louise Shuey and Geoff Pegg collected the sample material. I analysed the data, drafted the manuscript, and prepared the figures. Caryn Oates and Geoff Pegg helped draft the manuscript. Sanushka Naidoo and Louise Shuey conceived of and supervised the study as well as helped draft the manuscript. Sanushka Naidoo obtained the funding for the study.

3.1 Abstract

Austropuccinia psidii, commonly known as myrtle rust, is an obligate, biotrophic rust pathogen that causes rust disease on a broad host range of Myrtaceae species. *Eucalyptus grandis*, a widely cultivated hardwood Myrtaceae species, shows a range of responses (from susceptible to resistant) to *A. psidii* infection. This pathogen threatens both their natural range and various forest plantations across the world. This study aimed to investigate the *A. psidii* transcriptomic responses in resistant and susceptible *E. grandis* at four time points. RNA-seq reads were mapped to the *A. psidii* reference genome to quantify expressed genes at 12-hours post inoculation (hpi), 1-, 2- and 5-days post inoculation (dpi). A total of eight hundred and ninety expressed genes were found, of which forty-three were candidate effector proteins. These included a rust transferred protein (RTP1), expressed in susceptible hosts at 5-dpi and a hydrolase protein expressed in both resistant and susceptible hosts over time. Functional categorisation of expressed genes revealed processes enriched in susceptible hosts, including malate metabolic and malate dehydrogenase activity, implicating oxalic acid in disease susceptibility. These results highlight putative virulence or pathogenicity mechanisms employed by *A. psidii* to cause disease and provides the first insight into the molecular responses of *A. psidii* in *E. grandis* over time.

Keywords: oxalic acid; dual RNA-seq; phytohormones; host-pathogen interactions; *Eucalyptus*

3.2 Introduction

Austropuccinia psidii (Winter) Beenken (Beenken, 2017) is an obligate biotrophic rust pathogen that causes myrtle rust on a broad host range within Myrtaceae, affecting approximately 480 species within 86 genera (Soewarto et al., 2019). Myrtle rust is considered a global pandemic, with incidence reports in North America, South America, Asia, Africa and various Oceania countries (Marlatt and Kimbrough, 1979; Rayachhetry et al., 1997; Coutinho et al., 1998; Uchida et al., 2006; Kawanishi et al., 2009; Carnegie et al., 2010; Zhuang and Wei, 2011; Giblin, 2013; McTaggart et al., 2016; Roux et al., 2016; du Plessis et al., 2017; du Plessis et al., 2019). Myrtle rust causes significant damage to growing plant leaves and shoots, causing shoot tip dieback, stunted growth and in cases of severe infection, seedling death (Glen et al., 2007). Symptoms are known to vary within and between species, with some displaying complete resistance while others exhibit severe susceptibility (Minchinton et al., 2014).

Eucalyptus grandis is a highly important forestry species, revered for its wood quality and rapid growth properties (Grattapaglia et al., 2012). This economically and ecologically important hardwood species is vulnerable to various emerging pests and pathogens, including myrtle rust, causing widespread losses to the natural and economical range. *E. grandis* is considered highly susceptible to myrtle rust infection, although some variation in disease severity exists between different genotypes (Junghans et al., 2003a). Studies investigating the interactions of *E. grandis* and myrtle rust have revealed the importance of constitutive expression of defence-related genes in resistance (Santos et al., 2020). Constitutive expression of genes related to salicylic acid (SA)-mediated responses, photosynthesis and a plethora of leucine-rich receptors was linked to resistance against myrtle rust, while these responses were limited or absent in susceptible samples (Santos et al., 2020). A recent study combining proteomics and metabolomics to investigate the interactions between *A. psidii* and *E. grandis* implicated the phenylpropanoid pathway, photosynthetic pathway, and oxidative burst in the observed resistance against this pathogen (Sekiya et al., 2021). Plants susceptible to myrtle rust were found to exhibit similar responses, although earlier accumulation in resistant plants and more effective downstream control are the main factors separating the phenotypes (Sekiya et al., 2021). dos Santos et al. (2019) showed the importance of the cuticular waxes composition in the resistance against myrtle rust, with resistant plants having greater amounts of waxes than susceptible varieties. Moreover, susceptible *E. grandis* waxes contained hexadecenoic acid, and this compound was found to be favourable to *A. psidii*, impacting growth and germination.

There have been significant advances in our understanding of the mechanisms underlying host resistance against myrtle rust. Comparatively, few studies have investigated the mechanisms *A. psidii* employs to initiate host colonisation and disease. Previous studies have highlighted the importance of cell wall degrading enzymes (CWDEs) in the success of *A. psidii*, with enzymes such as peptidases, proteases, and modification proteins involved in host susceptibility (Quecine et al., 2016). Moreover, the authors found that susceptible guava infected with myrtle rust had greater abundance of pathogen-derived heat-shock proteins (HSPs), tubulin and actin proteins than what was found in the more resistant infected *Eucalyptus*, suggesting an involvement of these chaperones in maintaining pathogen virulence.

Rust fungi are a complex group of plant pathogens that consists of approximately 8000 species (Aime et al., 2017). These diverse fungal pathogens have significantly larger genomes than any other species, with sizes ranging from 300 Mbp to 2 Gbp (Aime et al., 2017; Bakkeren and Szabo, 2020). Due to the obligate biotrophic nature of rust fungi, there have been few studies on the molecular mechanisms governing the interactions with host plants (Bakkeren and Szabo, 2020). Studies on rust pathogens have revealed candidate effector proteins, although to date, few of these have been functionally characterised (Petre et al., 2014). A rust pathogen of poplar (*Melampsora larici-populina*) secretes an effector protein (Mlp124357) that increases susceptibility to bacterial and oomycete pathogens, suggesting the ability of this protein to manipulate host immunity (Madina et al., 2020). This protein was found to interact with a host protein to localise to the tonoplast of host cells to facilitate infection. Furthermore, this pathogen secretes an effector protein (Mlp37347) that localises to the plasmodesmata of host cells where it functions to reduce callose deposition to facilitate infection of neighbouring cells (Rahman et al., 2021). A rust transferred protein (RTP1) was identified from the broad bean rust pathogen, *Uromyces fabae* (Kemen et al., 2005) and found to form filaments inside the host plant to facilitate pathogen virulence during late-stage rust infection by protecting the haustorium from degradation (Kemen et al., 2013). Despite limited understanding of fungal effectors, and even more so of rust fungal effectors, recent advances in "omics" have facilitated studies on these complex organisms to unravel the role these proteins play during host colonisation and fungal proliferation.

There have been few studies investigating the molecular interactions of myrtle rust within its host plants, highlighting the need for resources that can advance our understanding of the mechanisms by which this pathogen causes disease. With the release of the *A. psidii* reference genome (Tobias et al., 2021), it is expected that many studies will emerge investigating this pathosystem. The identification of candidate effectors and virulence and pathogenicity genes highlights targets for future functional studies. The aim of this study was to investigate *A. psidii* responses in both resistant and susceptible

E. grandis, to identify candidate effector proteins as well as pathways involved in the interactions. We hypothesize greater expression of *A. psidii* genes in the susceptible compared to resistant hosts with the number of transcripts increasing over time (Hsieh et al., 2018). Elucidating the molecular mechanisms that govern these interactions will highlight novel pathogen targets for disease control and management. This is the first study to look at the molecular interactions of myrtle rust with *E. grandis* over a time series.

3.3 Materials and Methods

3.3.1 *Austropuccinia psidii* inoculation trial

Eucalyptus grandis seedlings were sourced from wild plants across their natural distribution in eastern Australia, ranging from Coffs Harbour in New South Wales to northern tropical regions of Queensland. Seedlings were grown from seed and germinated in glasshouse conditions, where temperatures ranged from 20-30ºC. The seedlings, all with at least four young leaves, were initially inoculated to determine the phenotypes. The inoculations were as reported in Swanepoel et al. (2021). In brief, spores of *A. psidii* were collected from *Syzygium jambos* growing as street trees in suburbs of Brisbane, Queensland. The spores were desiccated in silica gel for 48 h and stored at -80ºC. The spores were mixed with sterile distilled water with 0.05% tween 20 and spore counts were determined using a haemocytometer (concentration adjusted to 1×10^5 spore mL^{-1}). *E. grandis* seedlings were spray inoculated, with spores applied to the upper and lower surfaces of the leaves. The seedlings were screened for resistance and susceptibility on a scale of 1 to 5 two weeks post inoculation, where seedlings rated one were considered resistant (R-interaction) and seedlings rated five were considered susceptible (S-interaction), based on the system used in Junghans et al. (2003b).

Seedlings rated R and S were selected and diseased tissues were removed. The seedlings were allowed to reshoot for eight weeks. Seedlings were inoculated as described above (infected) or mock-inoculated with 0.05% tween 20 (control). Samples were collected from these seedlings at four time points (12-hpi, 1-, 2- and 5-dpi), with three replicates per time point and 14 seedlings per replicate.

3.3.2 RNA extraction, sequencing, and annotation

Total RNA extraction was performed on inoculated and uninoculated frozen leaf samples as described by Naidoo et al. (2013) and Swanepoel et al. (2021). The RNA samples were treated with Qiagen RNase-free DNase 1 enzyme (Qiagen Inc.). Extracted RNA was purified with Qiagen RNeasy Mini Kit as per the manufacturer's instruction. Samples were analysed on a Bio-Rad Experion analyser to

evaluate the RNA concentration and quality. Purified RNA from three biological replicates were submitted to the Beijing Genomics Institute (BGI) for mRNA-sequencing using 50 bp paired-end Illumina HiSeq 2500, an insert size of 300 bp and a sequencing depth of 40 million reads per sample.

To analyse the RNA-seq data, Galaxy Workspace (Goecks et al., 2010) was used to analyse the data and read quality was assessed using FASTQC v0.11.3. Adapter sequences were removed, and the integrity of the data was determined. The host (Myburg et al., 2014) and pathogen (Tobias et al., 2021) reference genomes were downloaded from Phytozome v12.1.5 (Goodstein et al., 2011) and Zenodo (DOI:10.5281/zenodo.3567172), respectively. The reference genomes were combined to create a genome index to be used in the mapping analysis. Quality filter-passed reads were mapped to the index genome using Spliced Transcript Alignment to a Reference (STAR), a universal RNA-seq aligner tool (Dobin et al., 2013) and read counts was determined using StringTie (Pertea et al., 2015). To obtain functional annotations of the myrtle rust genome, diamond v0.9.9 (Buchfink et al., 2015) was used to perform BLASTp similarity searches using the NCBI non-redundant protein database, RefSeq complete protein database and the UniProtKB/Swissprot database with a minimum-query coverage of 80%, minimum target coverage of 60% and a minimum e-value of 1e-05. To obtain functional gene descriptions and gene ontology (GO) terms for each *A. psidii* gene, EggNOG v0.99.1 (Huerta-Cepas et al., 2018) and InterProScan v5.25-64.0 (Jones et al., 2014) were used. To identify putative pathways involved in the *A. psidii* infection process, the protein sequences of the expressed *A. psidii* genes were annotated using GhostKOALA (Kanehisa et al., 2007) and analysed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database.

3.3.3 Differential expression analysis

To perform differential expression (DE) analysis, reads identified through transcript mapping were imported into R v1.4.1106 (R Core Team, 2018) using tximport v1.180 (Soneson et al., 2015). To identify confidently expressed *A. psidii* genes, transcripts with read counts lower than 20 in at least three libraries were filtered out, as they are considered lowly expressed. The imported, filtered read counts were analysed using DESeq2 v 1.30.1 (Love et al., 2014). Pathogen genes were considered significantly DE with a Benjamini & Hochberg FDR of $p < 0.05$ and when the absolute value of $\log_2$ fold change > 0.5. Comparisons between R- and S-interactions at each time point were made, where up-regulation refers to genes with greater expression in the S-interaction and down-regulation refers to genes with lower expression in the S-interaction.

3.3.4 Functional characterisation and identification of virulence and pathogenicity factors

To identify over-represented gene ontology (GO) biological process (BP), cellular component (CC) and molecular function (MF) *A. psidii* terms, DEGs separated into up- and down-regulated genes were used for GO enrichment using GOSeq v1.42.0 (Young et al., 2010) in R v1.4.1106, with a Benjamini & Hochberg false discovery rate of $p < 0.1$. Similarly, total expressed *A. psidii* genes separated into R- and S-interaction specific expression across the time series were used for GO enrichment following the same method. GO enrichment was determined separately for BP, CC, MF, and KEGG. To identify putative virulence and pathogenicity factors, expressed myrtle rust genes in the R- and S-interaction throughout the time series were identified and aligned the pathogen-host interaction (PHI) database v4.2 (Urban et al., 2020), with a minimum e-value of 1×10^{-4} and a minimum identity of 60% to the query sequence. Additionally, the expressed genes were compared to the candidate effectors identified by Tobias et al. (2021) in the R- and S-interaction throughout the time series. The protein sequences of the expressed candidate effectors were aligned to the NCBI non-redundant protein database with default parameters. Informative hits with a minimum e-value of 1×10^{-5} were selected for further analyses.

3.3.5 Gene clustering analyses

Variance stabilising transformation (VST) was applied to normalise the expression data using DESeq2 v1.30.1 (Love et al., 2014) across all samples and time points in the R- and S-interaction. Since the RNA was sequenced in different batches (12-hpi and 2-dpi; 1-dpi; 5-dpi), ComBat in sva v3.40.0 (Johnson et al., 2007; Leek et al., 2012) was used to remove the batch effect separately for the host and pathogen genes. Pathogen genes were clustered with a hierarchical clustering approach using a Euclidean distance metric and complete linkage clustering method to calculate the z-score using VST data. The number of clusters chosen to resolve the groups in the dendrogram cluster was K = 10. The z-score was used to generate line plots, where K = 10 and an expression heatmap across all samples.

3.3.6 Comparisons between *Austropuccinia psidii* transcriptome and proteome data

In a previous study investigating the proteome of *A. psidii* in susceptible guava and resistant *E. grandis,* urediniospores were collected from infected fruit and leaves of guava and *E. grandis,* respectively (Quecine et al., 2016). The authors determined the protein abundance of these samples and log ratios of susceptible relative to resistant abundance was made. We aimed to compare the proteome identified by Quecine et al. (2016) and the RNA-seq results obtained in the present study to gain a deeper understanding of these interactions. We obtained the protein accession numbers of the 340 total

detected proteins captured within the urediniospores and retrieved the protein sequences from the UniProtKB/Swiss (http://www.uniprot.org/ release Version 2021_04). We used the obtained protein sequences to perform a Conditional Reciprocal Best (CBR) BLAST (Aubry et al., 2014) against the predicted *A. psidii* proteome to identify hits that are the most likely representatives of the previously identified proteins. We compared these to the total expressed *A. psidii* genes to determine which proteins corresponded with expressed genes.

3.4 Results

3.4.1 Disease progression in *Eucalyptus grandis*

Disease progression was monitored for the presence of symptoms. Disease assessments were carried out as described by Swanepoel et al. (2021). Different responses were observed in the seedlings following the inoculations (Swanepoel et al., 2021). Briefly, symptoms ranged from light chlorotic flecking (rating one), visible HR and necrotic lesions (rating two), progression of necrosis and uredinia pustules (rating three and four) and formation of severe pustules in rating five.

3.4.2 RNA sequencing, mapping and expressed *A. psidii* genes

RNA-sequencing in both the R- and S-interactions yielded approximately 20 million paired-end reads per sample at each time point (Supplementary Table 3.1). As expected, 100% of reads in the control samples (con) mapped to the host reference genome for both R- and S-interactions at each time point, confirming the quality of the mapping analysis. Across the inoculated (inf) R- and S-interactions at each time point, ca. 99.9% of reads mapped to the host reference genome and ca. 0.09% of reads mapped to the pathogen reference genome. At 2- and 5-dpi in the resistant interaction, reads at one and two biological replicates, respectively, did not map to the pathogen reference genome. Similarly, at 2-dpi in the susceptible interaction, reads did not map to the pathogen genome. The overall lowly mapped pathogen reads may affect downstream gene expression analysis, as the number of mapped reads may not accurately represent the *in planta* interaction between *E. grandis* and *A. psidii*. More reads mapped to the S-interaction at 5-dpi than the R interaction. This is expected, as the S-interaction has significantly more disease symptoms than that of the R-interaction, and this may correlate with greater fungal biomass as the disease progresses in the S-interaction, owing to the detection of more fungal RNA.

There were a total 890 confidently expressed *A. psidii* genes throughout the R- and S-interactions over the time series, with 683 having successful annotations (ca. 77%). Four hundred and twenty-four of

the annotated genes had hits to hypothetical proteins (ca. 62%) and 26 genes had hits to uncharacterised proteins (ca. 4%). The remaining genes had informative hits that may shed light on the interactions between myrtle rust and *E. grandis*. Figure 3.1 shows the distribution of expressed genes across the time series. There were more expressed genes in the S-interaction at 12-hpi, with 418 compared to 380 in the R-interaction. The number of expressed *A. psidii* genes increased in the R-interaction to 709, while only 639 genes were expressed in the S-interaction. By 2-dpi, the number of genes significantly decreased in the R-interaction while remaining relatively stable in the S-interaction. During late-stage infection at 5-dpi, the number of genes in the S-interaction rose to 888, representing ca. 99% of the total expressed genes. Comparatively, the number of genes expressed in the R-interaction decreased dramatically to 234.

When investigating ten most highly expressed *A. psidii* genes in the R- and S-interaction, seven genes were of the most highly expressed in both the R- and S-interactions (Supplementary Table 3.2 and 3.3, respectively). Four of these genes, two highly expressed in both interactions and two highly expressed in either the R- or S-interactions, are among the candidate effectors defined by Tobias et al. (2021). Moreover, seven of these genes, five of which are highly expressed in both interactions and two of which are highly expressed in only the R-interaction, are among the list of significantly DEGs at 5-dpi. Unfortunately, these genes had no successful annotations. It is imperative to determine the identity of these genes and the role they play during the interaction, as it may shed light on the pathogenicity and virulence of *A. psidii*. This is particularly true of the genes uniquely highly expressed in the S-interaction. Similarly, when investigating the top 100 most highly expressed genes, comparisons between the R- and S-interaction revealed 78 genes in common between interactions and 22 genes unique to either the R- or S-interaction.

When analysing the total expressed genes using KEGG, 529 genes (ca. 59%) had successful annotations. The functional categories with the most annotations included “genetic information processing” (ca. 47%), “carbohydrate metabolism” (ca. 8%), “cellular processes” (ca. 6%) and “energy metabolism” (ca. 5%). KEGG enrichment revealed involvement of “2-oxocarboxylic acid metabolism”, “biosynthesis of amino acids” and “glyoxylate and dicarboxylate metabolism” among others (Supplementary Table 3.4).

3.4.3 Differentially expressed *Austropuccinia psidii* genes

To determine the differences in gene expression between the R- and S-interactions, differential gene expression analysis was performed. *A. psidii* DEGs were considered up-regulated when expression was greater in the S-interaction compared to the R-interaction. Since there were only 890 confidently

expressed *A. psidii* genes, there were very few DEGs. No significantly DEGs were identified at 12-hpi and 2-dpi. At 1-dpi, APSI_H004.3230 was significantly DE between the R- and S-interactions, with expression lower in the S-interaction (log_2 (fold change) = -7.2). This gene did not have successful hits when functionally annotated, so putative functions are unknown. At 5-dpi, 11 genes were up-regulated with expression greater in the S-interaction and 15 genes were down-regulated with expression lower in the S-interaction. Supplementary Table 3.5 shows the annotations of the 26 significantly DEGs at 5-dpi.

3.4.4 Gene ontology enrichment analysis

GO enrichment analyses were performed to identify putative pathways involved in the interactions between *E. grandis* and *A. psidii*. There were no significantly over-represented terms upon GO enrichment when analysing the DE dataset. This is expected due to the small number of significantly DEGs. When analysing the total gene space, 188 over-represented GO BP terms were identified across the R- and S-interaction over the time series (Figure 3.2, Supplementary Table 3.6). These terms were predominantly associated with cellular processes, including terms such as translation, cellular protein metabolic process, and cellular biosynthetic process. These processes were shared among both the R- and S-interaction over time series.

Terms that were unique to the S-interaction included energy processes such as ATP synthesis, energy coupled proton transport and mitochondrial ATP synthesis. Additionally, oxoacid metabolic processes and organic acid metabolic processes were also unique to the S-interaction. Terms unique to the R-interaction included glyoxylate cycle and metabolic process, dicarboxylic acid biosynthesis and cellular aldehyde metabolic process. Twenty-three over-represented MF terms were identified across the R- and S-interactions. Terms unique to the S-interaction included malate dehydrogenase activity and saccharopine dehydrogenase activity and terms involved in transcription were unique to the R-interaction (Supplementary Figure 3.1). When investigating the CC category, 77 over-represented terms were identified. Terms involving cellular processes were over-represented, including transcription and translation (Supplementary Figure 3.2).

3.4.5 *Austropuccinia psidii* pathogenicity and virulence factors

The 890 confidently expressed myrtle rust genes were analysed using the PHI database v4.2 (Urban et al., 2020). Genes with hits greater than 60% identity to the subject query and those implicated in virulence and pathogenicity were retained for further analysis. The R-interaction had 24 genes with successful hits (Table 3.1) while the S-interaction had 32 genes with successful hits to the subject

query (Table 3.2). Hits unique to the S-interaction included a gene involved in cAMP signalling (*Gib2*, APSI_P008.17130, APSI_H002.12341) and a gene encoding a putative pyridoxal 5'-phosphate synthase subunit (*PdxS*, APSI_P009.17505). Additionally, while the R- and S-interaction shared common hits, the S-interaction had expression of more orthologs of certain genes. These included expression of an additional cyclophilin (*CPA1*, APSI_P005.10514), tubulin alpha-1 chain (*TUB1*, APSI_H010.14180), conserved actin protein (*ActA*, APSI_H003.4114), hypothetical protein (MGG_00383, APSI_H021.3806) and a beta2-tubulin housekeeping gene (APSI_H018.10108). Hits shared between resistant and susceptible interactions included heat shock proteins (HSPs), a transcription factor identified in *Magnaporthe oryzae* and a gene encoding 3-isopropylmalate dehydratase (Table 3.1 and 3.2).

Analysis of the candidate effector proteins identified by Tobias et al. (2021) revealed a total of 43 expressed genes in the present study (Supplementary Figure 3.3, Supplementary Table 3.7). The highest expressed effectors in the R- and S-interaction were 33 and 43, respectively. The S-interaction had seven uniquely expressed candidate effectors over the course of infection, two at 2-dpi and five at 5-dpi (Supplementary Figure 3.3, Supplementary Table 3.7). The expression of these genes was not observed in the R-interaction. To investigate putative virulence and pathogenicity of these candidates, the protein sequences were subjected to a BLASTp on the NCBI non-redundant database. Seventeen of the 43 expressed genes had successful hits to proteins from other organisms, predominantly rust fungi. Many of these hits corresponded with hypothetical or uncharacterised proteins (Supplementary Table 3.8). Interestingly, five hits corresponded with informative hits (Table 3.3). A small subunit ribosomal protein S10e (APSI_H017.8250), a rust transferred protein 1 (*RTP*1, APSI_P008.18155) and a non-catalytic module family protein (APSI_H007.8820) was uniquely expressed in the S-interaction. A small subunit ribosomal protein S10e (APSI_P005.11212) and a hydrolase 76 protein (APSI_P004.3557) was expressed in both interactions. The expression of these genes in the R- and S-interaction across the time series is represented by Figure 3.3.

3.4.6 Clustering of expressed genes

To identify clusters of highly similar genes, based on expression profiles, hierarchical clustering was performed using a Euclidean distance metric and complete linkage clustering method. This provides information on the global changes in the transcriptome of *A. psidii* in resistant and susceptible *E. grandis* over the time series. The cluster dendrogram revealed three distinct clusters (Supplementary Figure 3.4). However, the number of genes in C1 and C2 was high, with 477 and 401 genes, respectively (Supplementary Figure 3.5). Considering there were a total of 890 genes, clusters of this

size will not be informative when performing downstream GO analyses, as the result from these analyses is not expected to differ from GO of the total gene space performed previously. Therefore, to resolve the lower groups into more distinct clusters to gain a deeper understanding of the genes underlying those groups, the number of clusters chosen for the analyses was ten. To visualise gene expression trends over the time series, line plots of the groups were generated for both the R- and S-interaction (Figure 3.4 and Supplementary Figure 3.5). This revealed interesting trends within the data. Clusters two, three, four, five, and nine have similar expression patterns during the early stages of infection in both the R- and S-interaction, but as the disease progresses, the expression of the genes within these clusters dramatically increases in the S-interaction when compared to the R-interaction. In contrast, the expression of genes within cluster eight increases significantly in the R-interaction at 5-dpi, while expression remains relatively stable at this time point in the S-interaction. Supplementary Table 3.9 shows the identity of the genes within each cluster, as well as their GO information. Similarly, the expression heatmap across the time series in both the R- and S-interaction revealed that expression patterns of *A. psidii* genes in both interactions are very similar during the early stages of infection at 12-hpi. As the course of infection progresses, the patterns become more varied over time between the R- and S-interaction (Supplementary Figure 3.6).

GO and KEGG enrichment was performed for each cluster identified in the hierarchical clustering analyses (Supplementary Table 3.10). Significant enrichment was identified in five clusters (C3 = 85; C4 = 49; C5 = 1; C7 = 2; C9 = 3). Enrichment in C3 included numerous terms associated with cellular processes such as cellular biosynthetic and metabolic processes, translation and mitochondrial-associated processes. Oxoacid metabolic process and tubulin complex were uniquely enriched in C3. Enrichment in C4 included terms associated with protein processing, including protein folding, protein metabolic process, and protein import into nucleus. Interestingly, response to heat was uniquely enriched in C4. Cluster 5 showed enrichment of 3-isopropylmalate dehydrogenase activity. C7 and C9 were enriched in endoplasmic reticulum insertion complex and ribosome-associated terms, respectively.

3.4.7 Comparisons of *Austropuccinia psidii* transcriptome and proteome

To gain a deeper understanding of the key mechanisms governing the interaction between *A. psidii* and its hosts, we compared the proteome results obtained by Quecine et al. (2016) to the RNA-seq results obtained in the present study. We identified 200 active and 140 obsolete entries (ca. 59%) for the proteins identified by Quecine et al. (2016) when retrieving protein sequences from UniProtKB/Swiss. This may be due to improvements in the genomes of the organisms, making some

entries obsolete or redundant. CRB BLAST results revealed 387 predicted *A. psidii* hits that are most likely representatives of the proteins identified in the proteome study (Supplementary Table 3.11). From this, 82 genes were expressed in our transcriptome study (Table 3.4, Supplementary Table 3.12). These included a plethora of HSPs that were either unique to susceptible guava (*P. guajava*) or in greater abundance in guava relative to *E. grandis*. There was greater expression of these HSPs in the R-interaction at 1-dpi, with expression in the S-interaction greater at 5-dpi. Other genes identified included those encoding for calnexin, enolase, pyruvate kinase, spermidine synthase and tubulin beta chain proteins. Many hypothetical proteins were identified within our dataset, with these uniquely abundant in guava or *E. grandis* (Table 3.4).

3.5 Discussion

Due to the obligate biotrophic nature of rust fungi, there have been limited studies on molecular genetics underlying the pathogen molecular dialogue with the host plant. With improving next generation sequencing, omics studies have facilitated the study of these complex organisms, highlighting candidate pathogenicity genes that can be studied using heterologous systems (Bakkeren and Szabo, 2020). This has broadened our understanding of rust disease and aided in development of efficient control strategies. The present study investigated the molecular responses of *A. psidii* in resistant and susceptible *E. grandis* over four time points. This revealed expression in both host types were similar, with various shared responses over time. Despite this, key differences between hosts were observed, including unique expression of candidate effectors in susceptible hosts as well as pathogenicity and virulence factors and pathways potentially contributing to disease. The results of this study are similar to that of Quecine et al. (2016), revealing that *A. psidii* might employ similar mechanisms to elicit host disease in different plant species.

Over the course of infection, the number of reads mapping to the pathogen genome decreased in the R-interaction. This suggests that over the course of infection, the R-interaction successfully suppresses the growth and development of the pathogen, thereby reducing the number of pathogen transcripts observed in the analysis. The number of transcripts mapping to the reference genome in the S-interaction significantly increases over the course of infection, suggesting that the S-interaction does not mount an effective defence response to prevent the proliferation of the pathogen. This is observed in the interaction between *A. psidii* and *M. quinquenervia*, where the number of transcripts mapping to the resistant hosts was 0% compared to 2% in the susceptible hosts, suggesting only susceptible hosts facilitate the growth of the pathogen at 5-dpi (Hsieh et al., 2018). This is observed in the present study, where the greatest number of expressed genes in the analysis were identified in the S-interaction

at 5-dpi (n = 888). The differences in mapping between *E. grandis* and *M. quinquenervia* might be indicative of varying host resistances against myrtle rust. These results are further supported by Tobias et al. (2018) in which resistant hosts actively respond to *A. psidii* infection, while susceptible hosts lack a sufficient, coordinated response, potentially contributing to the number of transcripts observed. This corroborates the results obtained in our previous studies on *E. grandis* responses to *A. psidii* (Swanepoel et al., 2021).

3.5.1 Shared virulence and pathogenicity factors

Cell wall degrading enzymes (CWDE) are enzymes employed by pathogens to degrade preformed barriers. In the present study, analysis of expressed candidate effectors revealed a family 76 hydrolase protein expressed in both the R- and S-interaction across the time series. Furthermore, a putative family 61 glycoside hydrolase protein was DE at 5-dpi, with expression significantly greater in the R- compared to the S-interaction. Hydrolase proteins are known to contribute to degradation of plant cell walls in other rust fungi (Cooper et al., 2016; Wu et al., 2019). Greater expression of these genes in the R-interaction is unexpected. It is possible that the pathogen is over-expressing these CWDE in resistant hosts to compensate for the effective preformed barriers that are preventing pathogen entry into the host plant (dos Santos et al., 2019). In susceptible hosts, entry into the plant cells occurs with relative ease and more rapidly, as preformed barriers are not adequate to prevent pathogen entry. Despite this, future studies will need to investigate this. These results suggest that *A. psidii* secretes a concoction of potent CWDE to facilitate the breakdown of the cell walls to allow successful host penetration.

Among the genes found in the interaction were numerous HSPs. HSPs are known to be involved in chaperoning the folding of proteins, but they also function to protect the cell from stress, including heat stress, fluctuations in pH and oxidative stress (Tiwari et al., 2015; Pandey et al., 2018). Two HSPs 90 (APSI_P013.4275, APSI_H009.11612) were identified as potential virulence factors in both the R- and S-interaction when compared to PHI-base. HSP90 is involved in complex protein folding processes and is vital to the functioning of the organism (Nathan et al., 1997). In *Fusarium graminearum,* HSP90 is required for pathogen virulence, with knockout mutants unable to spread from the initial infection site (Bui et al., 2016). This protein is highlighted as an antifungal target due to the function of HSP90 in crucial cellular processes including complex protein folding and the roles it plays in cellular morphology (Tiwari et al., 2015).

Previous studies on rust fungi virulence and pathogenicity factors have identified a plethora of HSPs enriched in susceptible hosts, suggesting that these proteins play crucial roles in facilitating plant

disease (Cooper et al., 2016; Quecine et al., 2016). Quecine et al. (2016) identified various HSPs during the interactions with *A. psidii* and resistant and susceptible hosts. More HSPs were identified in susceptible guava (*P. guajava*) than in resistant *E. grandis*, with the authors suggesting *A. psidii* is under more stressful conditions in susceptible hosts, for example oxidative stress due to production of ROS as a defence response. This is further suggested by Song et al. (2011), where a plethora of HSPs were isolated from the haustoria of *Puccinia triticina,* a wheat leaf rust fungus. The presence of these HSPs in the haustoria indicate the pathogen is under stress due to host defences, and these proteins could serve to protect the pathogen from host degradation (Song et al., 2011). The expression of HSPs in this study suggests a significant role of these proteins in the interaction between *A. psidii* and *E. grandis*. Furthermore, response to heat stress was enriched in cluster 4, and this cluster showed similar expression during initial stages of infection and dramatic increase of expression at 5-dpi. These proteins had greater expression in the R-interaction at 12-hpi and 1-dpi (Table 3.4, Supplementary Table 3.12), suggesting that the pathogen is under stress during the early interaction with resistant hosts, a possible response to host defences. Similar responses are only observed in the S-interaction at 2- and 5-dpi. Further studies are required to determine how these proteins facilitate disease and why expression is similar between resistant and susceptible *E. grandis*, despite contradicting evidence suggested by previous studies.

In the present study, we found one and two cyclophilin genes in the R- and S-interaction, respectively, when investigating virulence and pathogenicity factors in PHI base (Table 3.1 and 3.2, respectively). Mutant phenotypes of these genes resulted in reduced virulence in *Oryctolagus cuniculus* (rabbit; PHI:213) and *Mus musculus* (house mouse; PHI:213). Cyclophilins are a group of highly conserved proteins present in eukaryotes and prokaryotes (Handschumacher et al., 1984). These proteins play a diverse role in many cellular processes, but we know little about their roles in phytopathogenic pathogenicity. In *M. oryzae,* a biotrophic blast pathogen of rice, cyclophilins are known to affect appressoria turgor and lipid biosynthesis, acting as a virulence factor (Singh et al., 2018). In *Botrytis cinerea,* a grey mould necrotrophic pathogen, a cyclophilin was identified and implicated in virulence due to the reduction in disease symptoms in mutant strains (Viaud et al., 2003). Cyclophilin proteins were isolated from the haustoria of *P. triticina* during the interactions with wheat (Song et al., 2011), suggesting a virulence role of these proteins in rust pathogens.

Amino acid biosynthesis and metabolism pathways were significantly enriched in both the R- and S-interactions. These were also enriched in clusters 3, 4 and 5 (Supplementary Table 3.10). Enzyme 3-isopropylmalate dehydratase was enriched in cluster 5 and identified as a putative pathogenicity factor when compared to the PHI-base in both interactions (Table 3.1 and 3.2). Moreover, one of these

proteins were identified in the urediniospores of guava (*P. guajava)* and *E. grandis,* where abundance was greater is susceptible guava (*P. guajava)* than in resistant *E. grandis* (log ratio = 0.81, Table 3.4, Supplementary Table 3.12). This enzyme is involved in the biosynthesis of leucine. In *M. oryzae,* mutants deficient in other enzymes in the leucine biosynthesis pathway showed reduced pathogenicity, suggesting that the production of leucine is crucial in the ability to cause disease (Que et al., 2020). Furthermore, this enzyme was identified in the proteome of *A. psidii* in infected guava (*P. guajava*) and *E. grandis,* where abundance was greater in susceptible guava (Quecine et al., 2016). This implicates this enzyme in the disease process of *A. psidii* and tags it as an important pathway for disease control strategies.

In addition to leucine biosynthesis, lysine biosynthesis and metabolism was identified throughout this study, highlighting it as an important process in the functioning of *A. psidii* (Figure 3.2, Table 3.1, Table 3.2, Supplementary Table 3.10). *Lys20*, encoding a homocitrate synthase enzyme, was identified as a virulence factor in both the R- and S-interaction (Table 3.1 and Table 3.2, respectively). This protein was identified in *M. oryzae,* where it was found to contribute to fungal conidiation, hyphal growth, infection, and pathogenicity (Zhang et al., 2014). This protein interacts with G-protein signalling proteins to regulate the production of lysine (Zhang et al., 2014). Moreover, Lys20 is involved in the first step of the lysine biosynthesis pathway (Kur et al., 2010). The last step in the biosynthesis of lysine is the enzyme saccharopine dehydrogenase. In *Cryptococcus neoformans,* this enzyme was found to contribute to fungal virulence when mutants were unable to survive *in vivo* (Kingsbury et al., 2004). This enzyme was enriched in the R-interaction at 1-dpi and in S-interaction at 1- and 2-dpi (Supplementary Figure 3.1). The activity of this enzyme only at 1-dpi in the R-interaction suggests that *A. psidii* is unable to complete the lysine amino acid biosynthesis process during late stages of infection. This may contribute to the inability of the pathogen to infect resistant hosts. The involvement of various steps in the biosynthesis of lysine highlights this pathway for novel disease control strategies.

Interestingly, APSI_H016.15346 is expressed in both the R- and S-interactions at each time point and is highly expressed in both interactions, with expression within the top ten most highly expressed genes. This gene is also significantly DE at 5-dpi between the interactions, with expression greater in the R-interaction than in the S-interaction over the time series ($\log_2$(fold change) = -1.56). Additionally, based on the effector criteria defined by Tobias et al. (2021), APSI_H016.15346 was identified as a candidate effector protein. However, this gene is not characterised and did not align with any known pathogenicity or virulence factor when subjected to a BLASTp in the PHI database. Moreover, there were no successful hits when subjected to a protein BLAST on the NCBI non-

redundant database. DE at 5-dpi, with expression greater in the R-interaction, suggests this gene is imperative to the infection process. Lower expression in the S-interaction may be a result of the disease severity of the susceptible hosts. It is possible that expression is over-compensated in the R-interaction to elicit disease and similar responses are not observed in the S-interaction due to the disease symptoms observed at this time point due to fungal success. Functional studies are required to elucidate the role this gene plays during the interactions of myrtle rust and *E. grandis* to make inferences on its pathogenicity and virulence.

3.5.2 Virulence and pathogenicity factors uniquely expressed in susceptible hosts

Gib2 was uniquely expressed in susceptible hosts. This protein regulates cyclic AMP (cAMP) through the interactions with two proteins, Ras1 and adenylyl cyclase (Cac1), in *Cryptococcus neoformans*, with *Gib2* mutants having reduced virulence (Wang et al., 2014). Moreover, overexpressing *Gib2* promoted cAMP levels in infected cells (Wang et al., 2014). In phytopathogens, cAMP signalling is involved in surface recognition, appressorium formation, conidiation, infectious growth and pathogenicity of *M. oryzae* (Zhang et al., 2011). The absence of expression of *Gib2* in the R-interaction may suggest that regulation of cAMP signalling is suppressed, while expression in the S-interaction may highlight this gene as a putative virulence factor involved in the interaction. The inability to successfully regulate this key process may contribute to reduced fungal success in resistant hosts.

PdxS was found to contribute to viability, stress tolerance and virulence of the gram-negative bacterial pathogen, *Actinobacillus pleuopneumoniae,* which causes pleuropneumonia respiratory disease (Xie et al., 2017). *PdxS* catalyses the production of pyridoxal 5'-phosphate (PLP), a biochemically active form of vitamin B6 (Eliot and Kirsch, 2004). *PdxS* mutants exhibited abnormal morphology, with craters on their surfaces, suggesting that adequate production of PLP by *PdxS* is required for normal cell morphology (Xie et al., 2017). Mutants of *PdxS* had significantly reduced virulence of *A. pleuropneumoniae* in mouse models (Xie et al., 2017). PLP is a cofactor for phosphorylation, playing a key role in many physiological processes, including amino acid biosynthesis and metabolism. The absence of expression of *PdxS* in the R-interaction may lead to reduced PLP biosynthesis in resistant hosts, potentially contributing to the reduced virulence of *A. psidii*. In a study conducted by Song et al. (2011) on *P. triticina,* a Pdx1 protein was isolated from the haustoria during interactions with wheat. Moreover, a pyridoxine biosynthesis protein was more abundant in susceptible guava (*P. guajava)* than resistant *Eucalyptus* when investigating the proteome of *A. psidii* infected hosts (Quecine et al., 2016), suggesting that this pathway is involved in host susceptibility in some way.

3.5.3 Rust transferred protein 1 may manipulate the reactive oxygen species production

Five expressed *A. psidii* candidate effector genes had successful annotations when subjected to BLAST analysis (Table 3.3). A rust transferred protein (RTP1) was identified in the S-interaction at 5-dpi, with expression of this gene not detected in the R-interaction. This protein, initially identified in *Uromyces fabae,* was found to localise in the extra-haustoria matrix during early stages of infection as well as inside the host cell cytoplasm as disease progresses and the haustoria matures. This suggests RTP1p plays a crucial role in maintaining the biotrophic lifestyle with host plants (Kemen et al., 2005; Kemen et al., 2013). It was found that as the haustoria matures over the course of infection, high concentrations of RTP1p can be found within the host cytoplasm (Kemen et al., 2013). As a result, cyclosis of host nucleus and chloroplasts is inhibited, with the authors suggesting this cessation is the result of accumulation of RTP1p (Kemen et al., 2013).

The chloroplast plays an important role in the defence response by producing ROS (Kretschmer et al., 2020). By inhibiting the movement of chloroplast and causing accumulation at the extra-haustoria matrix, it reduces the photosynthetic capabilities of the chloroplast (Kemen et al., 2013). In turn, this reduces the production of ROS which may affect the ability of the host to undergo programmed cell death to limit the spread of the pathogen from the initial site of infection. Thus, accumulation of RTP1 may result in reduced downstream production of ROS. Consistent with previous studies, this protein was expressed during late stages of infection in susceptible *E. grandis* in response to infection (Kemen et al., 2005; Puthoff et al., 2008; Fernandez et al., 2012). This suggests RTP1p is required for pathogenicity of *A. psidii*.

3.5.4 Oxalic acid may manipulate host oxidative and phytohormone pathways

Malate dehydrogenase is an enzyme that catalyses the reaction of malate to oxaloacetate, a precursor molecule for oxalic acid production. The accumulation of oxalic acid produces an acidic environment within the host plant to facilitate crucial fungal mechanisms of infection, which includes the secretion of virulence factors (Lovat et al., 2019). Oxalic acid is known to reduce plant oxidative burst responses to facilitate *Sclerotinia sclerotiorum* disease in host plants (Cessna et al., 2000). Moreover, oxidative burst responses are reduced in acidic environments (Laurent et al., 1993). In the interaction between *Castanea* spp. and the chestnut blight pathogen *Cryphonectria parasitica,* oxalate (oxalic acid) is produced by the pathogen as a virulence factor to promote plant disease. This is through oxalate reducing the host cellular pH to promote the functioning of crucial fungal enzymes (Lovat et al., 2019). Researchers have exploited this fungal virulence factor by generating transgenic Chestnut (*C. dendata*)

with an oxalate oxidase gene that functions to convert oxalic acid to hydrogen peroxide and carbon dioxide (Zhang et al., 2013). This has shown to provide tolerance in *C. dendata* to *C. parasitica* infection and provides a promising control strategy in the interaction between myrtle rust and *E. grandis*.

In our previous study, we found the extensive involvement of phytohormones in the host responses to *A. psidii* (Swanepoel et al., 2021). This included salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) enriched in both the R- and S-interactions, as well as brassinosteroids (BR) enriched only in the R-interaction. In a study that investigated the oxalic acid-mediated stress in *Brassica napus* L., it was found that applications of this acid affected the phytohormone signalling and oxidative responses (Liang et al. 2009). Proteins associated with phytohormone pathways including JA and ET were increased following applications of oxalic acid. Despite the evidence suggesting that SA is not directly affected in the presence of oxalic acid, it was found that pathways mediated by the phytohormone were decreased (Liang et al., 2009). This suggests that oxalic acid might play a role in manipulating phytohormone pathways, thereby affecting the crosstalk that exists between them. This may account for the significant involvement of atypical phytohormones such as JA and ET in the interaction between *E. grandis* and *A. psidii*. These results may also contribute to the reduced BR signalling noted within the S-interaction, as an altered crosstalk may reduce the phytohormone signalling in the interaction.

In the present study, malate dehydrogenase was enriched in the S-interaction at 2- and 5-dpi (Supplementary Figure 3.1). Two malate dehydrogenase genes were identified when comparing transcriptome and proteome data (Quecine et al., 2016), as seen in Table 3.4, with abundance of these proteins greater in susceptible guava. Oxoacid metabolic processes were enriched in both the R- and S-interaction at 1- and 5-dpi, respectively (Figure 3.2), with oxalic acid forming part of oxoacids. Oxoacids were enriched in cluster 3, where expression of these genes was significantly greater at 5-dpi in the S-interaction. Malate metabolic processes were enriched in the S-interaction at 2- and 5-dpi (Figure 3.2). The enrichment of these terms suggests a putative role for this pathway in the virulence of *A. psidii.* In our previous study we found significant involvement of the oxidative burst response in defence against *A. psidii,* in both the R- and S-interaction (Swanepoel et al., 2021). The plant-type HR was prominent in resistant hosts at 2- and 5-dpi while susceptible hosts only responded with HR at 5-dpi. This suggests that despite both hosts regulating and mounting respiratory burst responses, the susceptible hosts lacked the ability to convert these into HR. This may be due to the involvement of pathogen secreted virulence factors, such as malate dehydrogenase and oxalic acid. This highlights a candidate pathway for control of myrtle rust infection.

3.6 Conclusions

While comparisons between the proteome (Quecine et al., 2016) and the transcriptome of *A. psidii* provides valuable insights into the molecular mechanisms governing the interactions of *A. psidii* with its hosts, it is important to remember that timing of collection of materials for analysis plays a role in the outcome of the results obtained. Furthermore, the study conducted on the proteome isolated urediniospores of *P. guajava* and *E. grandis*, while the present study isolated fungal RNA from whole leaf samples. Different infection structures and stages can affect the results obtained. Additionally, Quecine et al. (2016) considered the differences that exist between different species, while the present study aimed to determine the differences that exist within *E. grandis* provenances. Therefore, the results obtained in the comparisons need to be further validated to confirm the proteins and genes found contribute to disease susceptibility.

The interaction between resistant and susceptible *E. grandis* and *A. psidii* share similarities, with the timing of infection crucial to the disease progression, highlighted by unique pathogen genes expressed solely in the S-interaction at 5-dpi. Several pathways were highlighted in this study, putatively contributing to the molecular dialogue with *E. grandis.* This is the first study to investigate the expression of *A. psidii* genes *in planta* over a time series. Through this, several candidate *A. psidii* genes have been highlighted for future functional studies that investigate their roles in the interaction with *E. grandi*s. Future studies will need to validate the results obtained in the present study. This includes measuring ROS levels in the R- and S-interaction to determine if increased infection contributes to decrease in ROS. In conclusion, this reveals genes and pathways that may be manipulated to control the devastating effects this pathogen has on native and introduced Myrtaceae species.

3.7 References

Aime, M.C., Mctaggart, A.R., Mondo, S.J., and Duplessis, S. (2017). "Phylogenetics and phylogenomics of rust fungi," in *Advances in Genetics,* eds. J.P. Townsend & Z. Wang. (Academic Press: Elsevier), 267-307.

Aubry, S., Kelly, S., Kümpers, B.M.C., Smith-Unna, R.D., and Hibberd, J.M. (2014). Deep evolutionary comparison of gene expression identifies parallel recruitment of trans-factors in two independent origins of C4 photosynthesis. *PLoS Genetics* 10**,** e1004365. doi: 10.1371/journal.pgen.1004365.

Bakkeren, G., and Szabo, L.J. (2020). Progress on molecular genetics and manipulation of rust fungi. *Phytopathology* 110**,** 532-543. doi: 10.1094/PHYTO-07-19-0228-IA.

Beenken, L. (2017). *Austropuccinia*: a new genus name for the myrtle rust *Puccinia psidii* placed within the redefined family Sphaerophragmiaceae (Pucciniales). *Phytotaxa* 297**,** 53-61. doi: 10.11646/phytotaxa.297.1.5.

Buchfink, B., Xie, C., and Huson, D.H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nature methods* 12**,** 59-60.

Bui, D.-C., Lee, Y., Lim, J.Y., Fu, M., Kim, J.-C., Choi, G.J., et al. (2016). Heat shock protein 90 is required for sexual and asexual development, virulence, and heat shock response in *Fusarium graminearum*. *Scientific Reports* 6**,** 28154. doi: 10.1038/srep28154.

Carnegie, A.J., Lidbetter, J.R., Walker, J., Horwood, M.A., Tesoriero, L., Glen, M., et al. (2010). *Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia. *Australasian Plant Pathology* 39**,** 463-466. doi: 10.1071/AP10102.

Cessna, S.G., Sears, V.E., Dickman, M.B., and Low, P.S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell* 12**,** 2191-2199. doi: 10.1105/tpc.12.11.2191.

Cooper, B., Campbell, K.B., Beard, H.S., Garrett, W.M., and Islam, N. (2016). Putative rust fungal effector proteins in infected bean and soybean leaves. *Phytopathology* 106**,** 491-499. doi: 10.1094/PHYTO-11-15-0310-R.

Coutinho, T.A., Wingfield, M.J., Alfenas, A.C., and Crous, P.W. (1998). *Eucalyptus* Rust: A disease with the potential for serious international implications. *Plant Disease* 82**,** 819-825. doi: 10.1094/PDIS.1998.82.7.819.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* 29**,** 15-21. doi: 10.1093/bioinformatics/bts635.

dos Santos, I.B., Lopes, M.D.S., Bini, A.P., Tschoeke, B.a.P., Verssani, B.a.W., Figueredo, E.F., et al. (2019). The *Eucalyptus* cuticular waxes contribute in preformed defense against *Austropuccinia psidii*. *Frontiers in Plant Science* 9**,** 1978. doi: 10.3389/fpls.2018.01978.

Du Plessis, E., Granados, G.M., Barnes, I., Ho, W.H., Alexander, B.J.R., Roux, J., et al. (2019). The pandemic strain of *Austropuccinia psidii* causes myrtle rust in New Zealand and Singapore. *Australasian Plant Pathology* 48**,** 253-256. doi: 10.1007/s13313-019-0624-x.

Du Plessis, E., Mctaggart, A.R., Granados, G.M., Wingfield, M.J., Roux, J., Ali, M.I.M., et al. (2017). First report of myrtle rust caused by *Austropuccinia psidii* on *Rhodomyrtus tomentosa* (Myrtaceae) from Singapore. *Plant Disease* 101**,** 1676. doi: 10.1094/PDIS-04-17-0530-PDN.

Eliot, A.C., and Kirsch, J.F. (2004). Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. *Annual Review of Biochemistry* 73**,** 383-415. doi: 10.1146/annurev.biochem.73.011303.074021.

Fernandez, D., Tisserant, E., Talhinhas, P., Azinheira, H., Vieira, A., Petitot, A.S., et al. (2012). 454-pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* reveals *in planta*-expressed pathogen-secreted proteins and plant functions in a late compatible plant-rust interaction. *Molecular Plant Pathology* 13**,** 17-37. doi: 10.1111/j.1364-3703.2011.00723.x.

Giblin, F. (2013). Myrtle rust report: New Caledonia. *University of the Sunshine Coast, Maroochydore, Queensland*.

Glen, M., Alfenas, A.C., Zauza, E.a.V., Wingfield, M.J., and Mohammed, C. (2007). *Puccinia psidii*: A threat to the Australian environment and economy - A review. *Australasian Plant Pathology* 36**,** 1-16. doi: 10.1071/AP06088.

Goecks, J., Nekrutenko, A., Taylor, J., and Team, G. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome biology* 11**,** R86. doi: 10.1186/gb-2010-11-8-r86.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., et al. (2011). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Research* 40**,** D1178-D1186. doi: 10.1093/nar/gkr944.

Grattapaglia, D., Vaillancourt, R.E., Shepherd, M., Thumma, B.R., Foley, W., Külheim, C., et al. (2012). Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. *Tree Genetics & Genomes* 8**,** 463-508. doi: 10.1007/s11295-012-0491-x.

Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J., and Speicher, D.W. (1984). Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* 226**,** 544-547. doi: 10.1126/science.6238408.

Hsieh, J.-F., Chuah, A., Patel, H.R., Sandhu, K.S., Foley, W.J., and KüLheim, C. (2018). Transcriptome profiling of *Melaleuca quinquenervia* challenged by myrtle rust reveals differences in defense responses among resistant individuals. *Phytopathology* 108**,** 495-509. doi: 10.1094/PHYTO-09-17-0307-R.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S.K., Cook, H., et al. (2018). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research* 47**,** D309-D314. doi: 10.1093/nar/gky1085.

Johnson, W.E., Li, C., and Rabinovic, A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 8**,** 118-127. doi: 10.1093/biostatistics/kxj037.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., Mcanulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics (Oxford, England)* 30**,** 1236-1240. doi: 10.1093/bioinformatics/btu031.

Junghans, D.T., Alfenas, A.C., Brommonschenkel, S.H., Oda, S., Mello, E.J., and Grattapaglia, D. (2003a). Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. *Theoretical and Applied Genetics* 108**,** 175-180. doi: 10.1007/s00122-003-1415-9.

Junghans, D.T., Alfenas, A.C., and Maffia, L.A. (2003b). Escala de notas para quantificação da ferrugem em Eucalyptus. *Fitopatologia Brasileira* 28**,** 184-188.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2007). KEGG for linking genomes to life and the environment. *Nucleic Acids Research* 36**,** 480-484. doi: 10.1093/nar/gkm882.

Kawanishi, T., Uematsu, S., Kakishima, M., Kagiwada, S., Hamamoto, H., Horie, H., et al. (2009). First report of rust disease on ohia and the causal fungus, *Puccinia psidii*, in Japan. *Journal of General Plant Pathology* 75**,** 428-431. doi: 10.1007/s10327-009-0202-0.

Kemen, E., Kemen, A., Ehlers, A., Voegele, R., and Mendgen, K. (2013). A novel structural effector from rust fungi is capable of fibril formation. *Plant Journal* 75**,** 767-780. doi: 10.1111/tpj.12237.

Kemen, E., Kemen, A.C., Rafiqi, M., Hempel, U., Mendgen, K., Hahn, M., et al. (2005). Identification of a protein from rust fungi transferred from haustoria into infected plant cells. *Molecular Plant-Microbe Interactions* 18**,** 1130-1139. doi: 10.1094/mpmi-18-1130.

Kingsbury, J.M., Yang, Z., Ganous, T.M., Cox, G.M., and Mccusker, J.H. (2004). Novel chimeric spermidine synthase-saccharopine dehydrogenase gene (*SPE3-LYS9*) in the human pathogen *Cryptococcus neoformans*. *Eukaryotic Cell* 3**,** 752-763. doi: 10.1128/EC.3.3.752-763.2004.

Kretschmer, M., Damoo, D., Djamei, A., and Kronstad, J. (2020). Chloroplasts and plant immunity: where are the fungal effectors? *Pathogens* 9**,** 19. doi: 10.3390/pathogens9010019.

Kur, K., Gabriel, I., MorschhäUser, J., Barchiesi, F., Spreghini, E., and Milewski, S. (2010). Disruption of homocitrate synthase genes in *Candida albicans* affects growth but not virulence. *Mycopathologia* 170**,** 397-402. doi: 10.1007/s11046-010-9337-y.

Laurent, L., Susan, R., Heinstein, P.F., and Low, P.S. (1993). Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. *Plant Physiology* 102**,** 233-240. doi: 10.1104/pp.102.1.233.

Leek, J.T., Johnson, W.E., Parker, H.S., Jaffe, A.E., and Storey, J.D. (2012). The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 28**,** 882-883. doi: 10.1093/bioinformatics/bts034.

Liang, Y., Strelkov, S.E., and Kav, N.N. (2009). Oxalic acid-mediated stress responses in *Brassica napus* L. *Proteomics* 9**,** 3156-3173. doi: 10.1002/pmic.200800966.

Lovat, C.-A., Donnelly, D.J., and DoğMuş-LehtijäRvi, H.T.B. (2019). Mechanisms and metabolomics of the host-pathogen interactions between Chestnut (*Castanea* species) and Chestnut blight (*Cryphonectria parasitica*). *Forest Pathology* 49**,** e12562. doi: 10.1111/efp.12562.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15**,** 550. doi: 10.1186/s13059-014-0550-8.

Madina, M.H., Rahman, M.S., Huang, X., Zhang, Y., Zheng, H., and Germain, H. (2020). A poplar rust effector protein associates with protein disulfide isomerase and enhances plant susceptibility. *Biology* 9**,** 294. doi: 10.3390/biology9090294.

Marlatt, R.B., and Kimbrough, J.W. (1979). *Puccinia psidii* on *Pimenta dioica* in south Florida. *Plant Disease Reporter* 63**,** 510-512.

Mctaggart, A.R., Roux, J., Granados, G.M., Gafur, A., Tarrigan, M., Santhakumar, P., et al. (2016). Rust (*Puccinia psidii*) recorded in Indonesia poses a threat to forests and forestry in South-East Asia. *Australasian Plant Pathology* 45**,** 83-89. doi: 10.1007/s13313-015-0386-z.

Minchinton, E.J., Smith, D., Hamley, K., and Donald, C. (2014). Myrtle rust in Australia. *Acta Horticulturae***,** 89-90. doi: 10.17660/ActaHortic.2014.1055.19.

Myburg, A.A., Grattapaglia, D., Tuskan, G.A., Hellsten, U., Hayes, R.D., Grimwood, J., et al. (2014). The genome of *Eucalyptus grandis*. *Nature* 510**,** 356. doi: 10.1038/nature13308.

Naidoo, R., Ferreira, L., Berger, D.K., Myburg, A.A., and Naidoo, S. (2013). The identification and differential expression of *Eucalyptus grandis* pathogenesis-related genes in response to salicylic acid and methyl jasmonate. *Frontiers in Plant Science* 4**,** 43. doi: 10.3389/fpls.2013.00043.

Nathan, D.F., Vos, M.H., and Lindquist, S. (1997). *In vivo* functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. *Proceedings of the National Academy of Sciences* 94**,** 12949-12956. doi: 10.1073/pnas.94.24.12949.

Pandey, V., Singh, M., Pandey, D., and Kumar, A. (2018). Integrated proteomics, genomics, metabolomics approaches reveal oxalic acid as pathogenicity factor in *Tilletia indica* inciting Karnal bunt disease of wheat. *Scientific Reports* 8. doi: 10.1038/s41598-018-26257-z.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.-C., Mendell, J.T., and Salzberg, S.L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature biotechnology* 33**,** 290-295. doi: 10.1038/nbt.3122.

Petre, B., Joly, D.L., and Duplessis, S. (2014). Effector proteins of rust fungi. *Frontiers in Plant Science* 5**,** 416-416. doi: 10.3389/fpls.2014.00416.

Puthoff, D.P., Neelam, A., Ehrenfried, M.L., Scheffler, B.E., Ballard, L., Song, Q., et al. (2008). Analysis of expressed sequence tags from *Uromyces appendiculatus* hyphae and haustoria and their comparison to sequences from other rust fungi. *Phytopathology* 98**,** 1126-1135. doi: 10.1094/PHYTO-98-10-1126.

Que, Y., Yue, X., Yang, N., Xu, Z., Tang, S., Wang, C., et al. (2020). Leucine biosynthesis is required for infection-related morphogenesis and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. *Current Genetics* 66**,** 155-171. doi: 10.1007/s00294-019-01009-2.

Quecine, M.C., Leite, T.F., Bini, A.P., Regiani, T., Franceschini, L.M., Budzinski, I.G.F., et al. (2016). Label-free quantitative proteomic analysis of *Puccinia psidii* uredospores reveals differences of fungal populations infecting *Eucalyptus* and guava. *PLoS One* 11**,** e0145343. doi: 10.1371/journal.pone.0145343.

R Core Team (2018). "R: A language and environment for statistical computing", in: *R Foundation for Statistical Computing, Vienna, Austria. URL* https://www.R-project.org/.).

Rahman, M., Madina, M.H., Plourde, M.B., dos Santos, K.C.G., Huang, X., Zhang, Y., et al. (2021). The fungal effector Mlp37347 alters plasmodesmata fluxes and enhances susceptibility to pathogen. *Microorganisms* 9**,** 1232.

Rayachhetry, M.B., Elliott, M.L., and Van, T.K. (1997). Natural epiphytotic of the rust *Puccinia psidii* on *Melaleuca quinquenervia* in Florida. *Plant Disease* 81**,** 831. doi: 10.1094/pdis.1997.81.7.831a.

Roux, J., Granados, G., Shuey, L., Barnes, I., Wingfield, M.J., and Mctaggart, A.R. (2016). A unique genotype of the rust pathogen, *Puccinia psidii,* on Myrtaceae in South Africa. *Australasian Plant Pathology* 45**,** 645-652. doi: 10.1007/s13313-016-0447-y.

Santos, S.A., Vidigal, P.M.P., GuimarãEs, L.M.S., Mafia, R.G., Templeton, M.D., and Alfenas, A.C. (2020). Transcriptome analysis of *Eucalyptus grandis* genotypes reveals constitutive overexpression of genes related to rust (*Austropuccinia psidii*) resistance. *Plant Molecular Biology*. doi: 10.1007/s11103-020-01030-x.

Sekiya, A., Marques, F.G., Leite, T.F., Cataldi, T.R., De Moraes, F.E., Pinheiro, A.L.M., et al. (2021). Network analysis combining proteomics and metabolomics reveals new insights into early responses of *Eucalyptus grandis* during rust infection. *Frontiers in Plant Science* 11. doi: 10.3389/fpls.2020.604849.

Singh, K., Winter, M., Zouhar, M., and RyšáNek, P. (2018). Cyclophilins: less studied proteins with critical roles in pathogenesis. *Phytopathology* 108**,** 6-14. doi: 10.1094/PHYTO-05-17-0167-RVW.

Soewarto, J., Giblin, F., and Carnegie, A.J. (2019). *Austropuccinia psidii (myrtle rust) global host list. Version 4* [Online]. Canberra, ACT: Australian Network for Plant Conservation. http://www.anpc.asn.au/myrtle-rust. Accessed 20 November 2020. Available: http://www.anpc.asn.au/myrtle-rust [Accessed 20 November 2020].

Soneson, C., Love, M.I., and Robinson, M.D. (2015). Differential analyses for RNA-seq: Transcript-level estimates improve gene-level inferences. *F1000Research* 4**,** 1521. doi: 10.12688/f1000research.7563.1.

Song, X., Rampitsch, C., Soltani, B., Mauthe, W., Linning, R., Banks, T., et al. (2011). Proteome analysis of wheat leaf rust fungus, *Puccinia triticina*, infection structures enriched for haustoria. *Proteomics* 11**,** 944-963. doi: 10.1002/pmic.201000014.

Swanepoel, S., Oates, C.N., Shuey, L.S., Pegg, G.S., and Naidoo, S. (2021). Transcriptome analysis of *Eucalyptus grandis* implicates brassinosteroid signaling in defense against myrtle rust (*Austropuccinia psidii*). *Frontiers in Forests and Global Change* 4**,** 778611. doi: 10.3389/ffgc.2021.778611.

Tiwari, S., Thakur, R., and Shankar, J. (2015). Role of heat-shock proteins in cellular function and in the biology of fungi. *Biotechnol Res Int* 2015**,** 132635-132635. doi: 10.1155/2015/132635.

Tobias, P.A., Guest, D.I., Külheim, C., and Park, R.F. (2018). De novo transcriptome study identifies candidate genes involved in resistance to *Austropuccinia psidii* (myrtle rust) in *Syzygium luehmannii* (riberry). *Phytopathology* 108**,** 627-640. doi: 10.1094/PHYTO-09-17-0298-R.

Tobias, P.A., Schwessinger, B., Deng, C.H., Wu, C., Dong, C., Sperschneider, J., et al. (2021). *Austropuccinia psidii,* causing myrtle rust, has a gigabase-sized genome shaped by transposable elements. *G3 Genes|Genomes|Genetics* 11**,** jkaa015. doi: 10.1093/g3journal/jkaa015.

Uchida, J., Zhong, S., and Killgore, E. (2006). First report of a rust disease on Ohia caused by *Puccinia psidii* in Hawaii. *Plant Disease* 90**,** 524. doi: 10.1094/pd-90-0524c.

Urban, M., Cuzick, A., Seager, J., Wood, V., Rutherford, K., Venkatesh, S.Y., et al. (2020). PHI-base: the pathogen–host interactions database. *Nucleic acids research* 48**,** D613-D620.

Viaud, M., Brunet-Simon, A., Brygoo, Y., Pradier, J.M., and Levis, C. (2003). Cyclophilin A and calcineurin functions investigated by gene inactivation, cyclosporin A inhibition and cDNA arrays approaches in the phytopathogenic fungus *Botrytis cinerea*. *Molecular Microbiology* 50**,** 1451-1465. doi: 10.1046/j.1365-2958.2003.03798.x.

Wang, Y., Shen, G., Gong, J., Shen, D., Whittington, A., Qing, J., et al. (2014). Noncanonical Gβ Gib2 is a scaffolding protein promoting cAMP signaling through functions of Ras1 and Cac1 proteins in *Cryptococcus neoformans**. *The Journal of Biological Chemistry* 289**,** 12202-12216. doi: 10.1074/jbc.M113.537183.

Wu, W., Nemri, A., Blackman, L.M., Catanzariti, A.-M., Sperschneider, J., Lawrence, G.J., et al. (2019). Flax rust infection transcriptomics reveals a transcriptional profile that may be indicative for rust Avr genes. *PloS one* 14**,** e0226106.

Xie, F., Li, G., Wang, Y., Zhang, Y., Zhou, L., Wang, C., et al. (2017). Pyridoxal phosphate synthases PdxS/PdxT are required for *Actinobacillus pleuropneumoniae* viability, stress tolerance and virulence. *PLoS One* 12**,** e0176374. doi: 10.1371/journal.pone.0176374.

Young, M.D., Wakefield, M.J., Smyth, G.K., and Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology* 11**,** R14.

Zhang, B., Oakes, A., Newhouse, A., Baier, K., Maynard, C., and Powell, W. (2013). A threshold level of oxalate oxidase transgene expression reduces *Cryphonectria parasitica*-induced necrosis in a transgenic American chestnut (*Castanea dentata*) leaf bioassay. *Transgenic Research* 22. doi: 10.1007/s11248-013-9708-5.

Zhang, H., Liu, K., Zhang, X., Tang, W., Wang, J., Guo, M., et al. (2011). Two phosphodiesterase genes, *PDEL* and *PDEH*, regulate development and pathogenicity by modulating intracellular cyclic AMP levels in *Magnaporthe oryzae*. *PloS One* 6**,** e17241. doi: 10.1371/journal.pone.0017241.

Zhang, H., Ma, H., Xie, X., Ji, J., Dong, Y., Du, Y., et al. (2014). Comparative proteomic analyses reveal that the regulators of G-protein signaling proteins regulate amino acid metabolism of the rice blast fungus *Magnaporthe oryzae*. *Proteomics* 14**,** 2508-2522. doi: 10.1002/pmic.201400173.

Zhuang, J., and Wei, S. (2011). Additional materials for the rust flora of Hainan Province, China. *Mycosystema* 30**,** 853-860.

3.8 Tables and figures

Table 3.1. Expressed *Austropuccinia psidii* PHI-annotated genes in the R-interaction with percentage identity greater than 60% and implications in pathogenicity and virulence.

Query identity	Name	PHI base gene description	Identity (%)	Mutant phenotype	PHI base accession
APSI_P002.15004	*Gsk3*	glycogen synthase kinase	70	0; 1	PHI:1200; PHI:4587
APSI_P020.4950	*ADE13*	Bifunctional enzyme adenylosuccinate (ADS) lyase	77	0	PHI:7186
APSI_P017.12437	*ActA*	Conserved actin protein	90	0	PHI:10434
APSI_P018.7518	Beta2-tubulin	Beta2-tubulin housekeeping gene	85	0	PHI:10566
APSI_P001.6093	Calcium permease	Ca2+ exchanger	61	1	PHI:2105
APSI_H012.10735	*CLA4*	Serine/threonine kinase	80	1	PHI:528
APSI_H008.9528	*CPA1*	Cyclophillin	68	0	PHI:213
APSI_P013.4275	*HSP90*	Ubiquitous chaperone, heat shock protein 90	70	0	PHI:6272
APSI_H009.11612	*HSP90*	Ubiquitous chaperone, heat shock protein 90	64	0	PHI:6272
APSI_P003.2172	*Leu1*	3-Isopropylmalate dehydratase	62	1	PHI:9357
APSI_P011.231	*ILV2*	Acetolactate synthase	65	1	PHI:358
APSI_P001.6880	MGG_00383	Hypothetical protein	74	0	PHI:877
APSI_H010.13828	*GSK1*	Glycogen synthase kinase, central signal regulator involved in the stress-responsive mechanism	69	1	PHI:7117
APSI_P014.1429	*Hox7*	transcription factor	62	1	PHI:2131
APSI_P002.14583	*LYS20*	regulators of G-protein (GTP-binding protein) signalling (RGS) proteins/homocitrate synthase	68	0	PHI:3234
APSI_H010.13601	*LYS20*	regulators of G-protein (GTP-binding protein) signalling (RGS) proteins/homocitrate synthase	64	0	PHI:3234
APSI_P001.5642	*Ssb1*	Heat shock protein	68	1	PHI:8179
APSI_P001.5636	*Ssb1*	Heat shock protein	68	1	PHI:8179
APSI_P015.13172	Pyruvate kinase	Pyruvate kinase	61	0; 1	PHI:8611
APSI_P001.5837	ScOrtholog_*PSR2*	Uncharacterised protein	70	0	PHI:5705
APSI_P002.15358	*TUB1*	Tubulin alpha-1 chain	74	1; 2	PHI:2530
APSI_H010.13652	*TUB1*	Tubulin alpha-1 chain	78	1; 2	PHI:2530
APSI_P015.13025	*TufM*	Mitochondrial elongation factor Tu	63	0	PHI:9772

Table 3.1 *(continued)*

APSI_P003.1647	*um01947*	Cytochrome C peroxidase precursor	63	0	PHI:854

0 = reduced virulence; 1 = loss of pathogenicity; 2 = lethal

Table 3.2. Expressed *Austropuccinia psidii* PHI-annotated genes in the S-interaction with percentage identity greater than 60% and implications in pathogenicity and virulence

Query identity	Gene name	PHI base gene description	Identity (%)	Mutant phenotype	PHI base accession
APSI_P002.15004	*Gsk3*	glycogen synthase kinase	70	0; 1	PHI:1200; PHI:4587
APSI_P020.4950	*ADE13*	Bifunctional enzyme adenylosuccinate (ADS) lyase	77	0	PHI:7186
APSI_H003.4114	*ActA*	Conserved actin protein	81	0	PHI:10434
APSI_P017.12437	*ActA*	Conserved actin protein	90	0	PHI:10434
APSI_H018.10108	Beta2-tubulin	Beta2-tubulin housekeeping gene	85	0	PHI:10566
APSI_P018.7518	Beta2-tubulin	Beta2-tubulin housekeeping gene	85	0	PHI:10566
APSI_P001.6093	Calcium permease	Calcium permease	61	0	PHI:2105
APSI_H012.10735	*CLA4*	Serine/threonine kinase	80	1	PHI:528
APSI_P005.10514	*CPA1*	Cyclophillin	68	0	PHI:213
APSI_H008.9528	*CPA1*	Cyclophillin	68	0	PHI:213
APSI_P013.4275	*HSP90*	Ubiquitous chaperone, heat shock protein 90	70	0	PHI:6272
APSI_H009.11612	*HSP90*	Ubiquitous chaperone, heat shock protein 90	64	0	PHI:6272
APSI_P003.2172	*Leu1*	3-Isopropylmalate dehydratase	62	1	PHI:9357
APSI_P008.17130	*Gib2*	Scaffolding Protein Promoting cAMP Signalling	80	0	PHI:4182
APSI_H002.12341	*Gib2*	Scaffolding Protein Promoting cAMP Signalling	80	0	PHI:4182
APSI_P011.231	*ILV2*	Acetolactate synthase	65	1	PHI:358
APSI_H021.3806	MGG_00383	Hypothetical protein	70	0	PHI:877
APSI_P001.6880	MGG_00383	Hypothetical protein	74	0	PHI:877
APSI_H010.13828	*GSK1*	Glycogen synthase kinase, central signal regulator involved in the stress-responsive mechanism	69	1	PHI:7117
APSI_P014.1429	*Hox7*	transcription factor	62	1	PHI:2131
APSI_P002.14583	*LYS20*	regulators of G-protein (GTP-binding protein) signalling (RGS) proteins/homocitrate synthase	68	0	PHI:3234
APSI_H010.13601	*LYS20*	regulators of G-protein (GTP-binding protein) signalling (RGS) proteins/homocitrate synthase	64	0	PHI:3234
APSI_P001.5642	*Ssb1*	Heat shock protein	68	1	PHI:8179
APSI_P001.5636	*Ssb1*	Heat shock protein	68	1	PHI:8179
APSI_P009.17505	*pdxS*	pyridoxal 5'-phosphate synthase subunit PdxS	67	0	PHI:7075
APSI_P015.13172	Pyruvate kinase	Pyruvate kinase	61	0; 1	PHI:8611
APSI_P001.5837	ScOrtholog_*PSR2*	Uncharacterised protein	70	0	PHI:5705
APSI_H010.14180	*TUB1*	Tubulin alpha-1 chain	76	1; 2	PHI:2530

Table 3.2 *(continued)*

APSI_P002.15358	*TUB1*	Tubulin alpha-1 chain	74	1; 2	PHI:2530
APSI_H010.13652	*TUB1*	Tubulin alpha-1 chain	78	1; 2	PHI:2530
APSI_P015.13025	*TufM*	Mitochondrial elongation factor Tu	63	0	PHI:9772
APSI_P003.1647	*um01947*	Cytochrome C peroxidase precursor	63	0	PHI:854

0 = reduced virulence; 1 = loss of pathogenicity; 2 = lethal

Table 3.3. Informative protein BLAST results for the expressed candidate *Austropuccinia psidii* effectors in resistant and susceptible *Eucalyptus grandis* identified on the non-redundant NCBI database.

Candidate effector	Accession	Identity (%)	Coverage (%)	Species	Description	E-value[a]
APSI_H017.8250	KNZ48236.1	82.2	82	*Puccinia sorghi*	Small subunit ribosomal protein S10e	5.49E-83
APSI_P005.11212	KNZ48236.1	81.5	82	*Puccinia sorghi*	Small subunit ribosomal protein S10e	3.55E-82
APSI_P004.3557	KAA1090934.1	75.9	75.9	*Puccinia graminis* f. sp. *triticina*	Hydrolase 76 protein	7.44E-136
APSI_P009.18155	AFI13823.1	43.8	100	*Gymnosporangium sabinae*	Rust transferred protein 1	2.13E-50
APSI_H007.8820	XP_007403659.1	38.7	78	*Melampsora larici-populina* 98AG31	Non-catalytic module family EXPN	1.23E-38

Table 3.4. Expressed *Austropuccinia psidii* genes from comparisons to proteomic data obtained by Quecine et al. (2016).

Gene ID	Description	Log ratio (ApG/ApE)[a]	Log fold change (SI/RI)[b]			
			12-hpi	1-dpi	2-dpi	5-dpi
APSI_H002.12538	-	-0.84	0.00	0.24	0.61	0.87
APSI_P002.14830	-	0.22	0.00	0.00	0.00	1.65
APSI_P015.13025	-	ApGuava	0.00	-3.09	0.19	0.70
APSI_P004.3474	3-isopropylmalate dehydrogenase	0.81	0.49	-0.71	-1.59	1.54
APSI_H009.11558	ATP synthase subunit alpha, mitochondrial	0.24	0.00	1.17	3.57	2.39
APSI_H005.1677	2-isopropylmalate synthase	0.21	-3.42	-0.60	0.00	0.70
APSI_P002.15726	2-methylcitrate dehydratase	0.3	2.80	-0.76	-0.41	0.85
APSI_P007.14326	3-isopropylmalate dehydrogenase	0.81	-3.33	0.19	0.32	-0.72
APSI_H004.3682	40S ribosomal protein S7	-0.38	0.00	0.00	3.11	2.80
APSI_H001.6084	Acetyl-CoA carboxylase	ApEucalyptus	1.56	1.18	-0.32	-0.75
APSI_H003.4114	Actin	0.09	1.73	0.00	0.00	1.15
APSI_P017.12437	Actin	0.09	0.00	-2.32	1.43	2.06
APSI_H007.8988	Adenosylhomocysteinase	-0.01	-2.84	0.00	-0.32	0.98
APSI_H014.2045	Arabinitol dehydrogenase 1	0.18	0.00	0.00	0.20	1.22
APSI_H015.423	Arginyl-tRNA synthetase	0.11	0.00	0.59	0.00	0.44
APSI_H004.3499	Aspartate aminotransferase, mitochondrial	0.31	0.00	2.45	2.38	1.00
APSI_P004.2874	Aspartate aminotransferase, mitochondrial	0.31	-4.63	-4.06	0.09	2.46
APSI_P011.364	ATP synthase subunit beta, mitochondrial	0.14	0.94	-1.98	0.08	2.33
APSI_H014.2180	Calnexin	-0.4	0.37	-2.34	0.00	0.86
APSI_P005.10461	Calnexin	-0.4	-0.76	-1.37	-0.49	2.34
APSI_P005.10288	Chlorophyll synthesis pathway protein	0.18	0.37	-1.44	0.00	0.74
APSI_P016.16045	Elongation factor 2	0	-1.55	-1.17	3.01	2.74
APSI_P016.16081	Elongation factor 2	0	0.00	-1.71	1.45	2.69
APSI_H008.9525	Enolase	0.21	0.00	2.44	0.00	0.72
APSI_P005.10519	Enolase	0.21	0.00	-2.32	1.20	1.47
APSI_P010.11580	Eukaryotic translation initiation factor 3 subunit F	0.13	-1.64	0.41	0.06	-0.35
APSI_H014.2368	Fatty acid synthase subunit beta	ApEucalyptus	0.00	0.00	0.00	0.00
APSI_P005.10846	Fatty acid synthase subunit beta	ApEucalyptus	-0.59	-1.69	-0.82	0.36
APSI_H003.4176	Glucose-regulated protein	0.08	0.00	1.86	0.00	0.42
APSI_P017.12534	Glucose-regulated protein	0.08	0.00	0.18	1.44	0.57
APSI_P008.16892	Glutamate dehydrogenase	0.14	0.00	-1.46	3.52	0.59
APSI_P010.11420	Heat shock 70kda protein 4	0.49	0.00	-3.15	2.06	2.69
APSI_P010.11427	Heat shock 70kda protein 4	0.49	0.56	-2.92	1.67	3.11
APSI_P013.4275	Heat shock protein 83	0.1	-1.53	-1.10	1.40	1.15
APSI_H022.14	Heat shock protein HSS1	0.08	-4.43	-0.32	4.02	2.33
APSI_P009.17506	Heat shock protein HSS1	0.08	-0.15	-0.54	1.38	2.63
APSI_P001.5636	Heat shock protein SSB	0.17	-3.65	0.01	1.50	3.38
APSI_P001.5642	Heat shock protein SSB	0.17	-1.73	-0.02	1.18	3.37
APSI_H009.11612	Heat-shock protein 90	0.1	-1.37	0.23	2.27	1.73
APSI_H001.6204	Hsp70-like protein	0.3	0.00	3.92	1.51	2.42
APSI_P002.14610	Hsp70-like protein	0.3	0.00	0.34	2.32	-0.08
APSI_P008.17020	Hypothetical protein	ApEucalyptus	1.16	-2.20	-0.03	0.54
APSI_H016.15678	Hypothetical protein	ApGuava	0.00	0.00	1.54	0.66
APSI_H001.6960	Hypothetical protein	0.43	0.00	1.86	2.17	0.10
APSI_P014.1140	Hypothetical protein	0.35	1.87	2.30	1.69	1.76

Table 3.4 *(continued)*

APSI_H003.4994	Hypothetical protein	-0.09	0.00	0.00	1.43	1.98
APSI_P014.1093	Hypothetical protein	-0.09	0.00	1.24	0.97	2.08
APSI_P001.5930	Hypothetical protein	ApEucalyptus	2.73	3.47	1.44	0.18
APSI_H018.10185	Hypothetical protein	ApGuava	1.96	0.00	-1.14	0.01
APSI_H016.15562	Hypothetical protein	0.36	0.00	-0.24	0.00	2.01
APSI_P010.11387	Hypothetical protein	-0.17	-0.38	0.88	-1.28	-0.94
APSI_H006.15165	Hypothetical protein	-0.05	-3.58	1.01	2.07	0.83
APSI_P009.17824	Hypothetical protein	ApGuava	0.00	-1.98	2.17	1.28
APSI_P006.9484	Hypothetical protein	ApGuava	0.00	0.00	0.92	1.06
APSI_P003.1597	Hypothetical protein	ApGuava	0.00	0.04	-1.04	0.56
APSI_P011.239	Hypothetical protein	-0.21	2.28	-1.36	-4.68	-0.79
APSI_P011.268	Hypothetical protein	0.53	3.14	-3.06	1.95	0.62
APSI_P019.8310	Hypothetical protein	0.43	0.00	0.00	1.54	0.10
APSI_P011.233	Hypothetical protein	ApGuava	0.00	0.05	2.88	1.73
APSI_P016.16382	Hypothetical protein	0.16	-0.51	-0.92	0.52	2.77
APSI_H017.8116	Hypothetical protein	0.03	0.00	-0.60	0.00	0.50
APSI_H017.8250	Hypothetical protein	ApGuava	0.00	0.00	0.00	1.22
APSI_P012.9011	Kinesin family member C1	0.06	1.45	1.05	1.40	2.81
APSI_H001.6292	Malate dehydrogenase, NAD-dependent	0.56	0.00	-1.26	2.08	1.75
APSI_P003.1614	Malate dehydrogenase, NAD-dependent	0.56	0.00	1.40	-0.49	1.72
APSI_H013.5798	Minichromosome maintenance protein 4	ApGuava	1.17	-1.35	1.58	-0.08
APSI_P010.11499	Minichromosome maintenance protein 6	0	-2.30	2.95	1.20	-0.37
APSI_P001.6720	Polyubiquitin-A	ApEucalyptus	-0.31	-0.22	-0.64	0.37
APSI_P017.12651	Polyubiquitin-A	ApEucalyptus	0.00	-1.44	0.00	1.41
APSI_H009.11705	Protein transporter SEC23	0.06	0.00	1.24	1.44	0.30
APSI_P013.4176	Protein transporter SEC23	0.06	0.00	2.49	0.91	0.40
APSI_H016.15523	Putative histone H9	ApEucalyptus	0.00	-3.77	1.43	0.39
APSI_P015.13172	Pyruvate kinase	ApGuava	-0.59	0.00	0.00	-0.29
APSI_P005.10979	RuvB-like helicase 1	ApGuava	0.37	-3.25	1.45	1.05
APSI_P016.16384	Secretory pathway GDP dissociation inhibitor 1	0.68	0.00	0.29	0.00	0.39
APSI_P018.7791	Spermidine synthase	ApGuava	0.00	1.07	0.31	0.29
APSI_P007.14016	T-complex protein 1 subunit alpha	-0.12	0.00	-1.62	0.00	-0.04
APSI_H006.15105	Translation initiation factor eIF-3	-0.11	0.00	0.00	0.00	0.28
APSI_H018.10108	Tubulin beta chain	0.34	0.00	0.00	0.00	0.72
APSI_P018.7518	Tubulin beta chain	0.34	0.00	-1.09	0.00	0.38
APSI_P002.15126	Uncharacterized protein	ApGuava	0.00	1.21	2.57	0.65
APSI_P007.13634	Vacuolar protein 8	ApGuava	-1.52	-0.54	-0.72	2.30

[a] Log ratio of ApGuava relative to ApEucalyptus (Quecine et al., 2016); [b] Log fold change of susceptible relative to resistant *E. grandis*

ApG = *Austropuccinia psidii* guava; ApE = *Austropuccinia psidii E. grandis;* SI = S-interaction; RI = R-interaction.

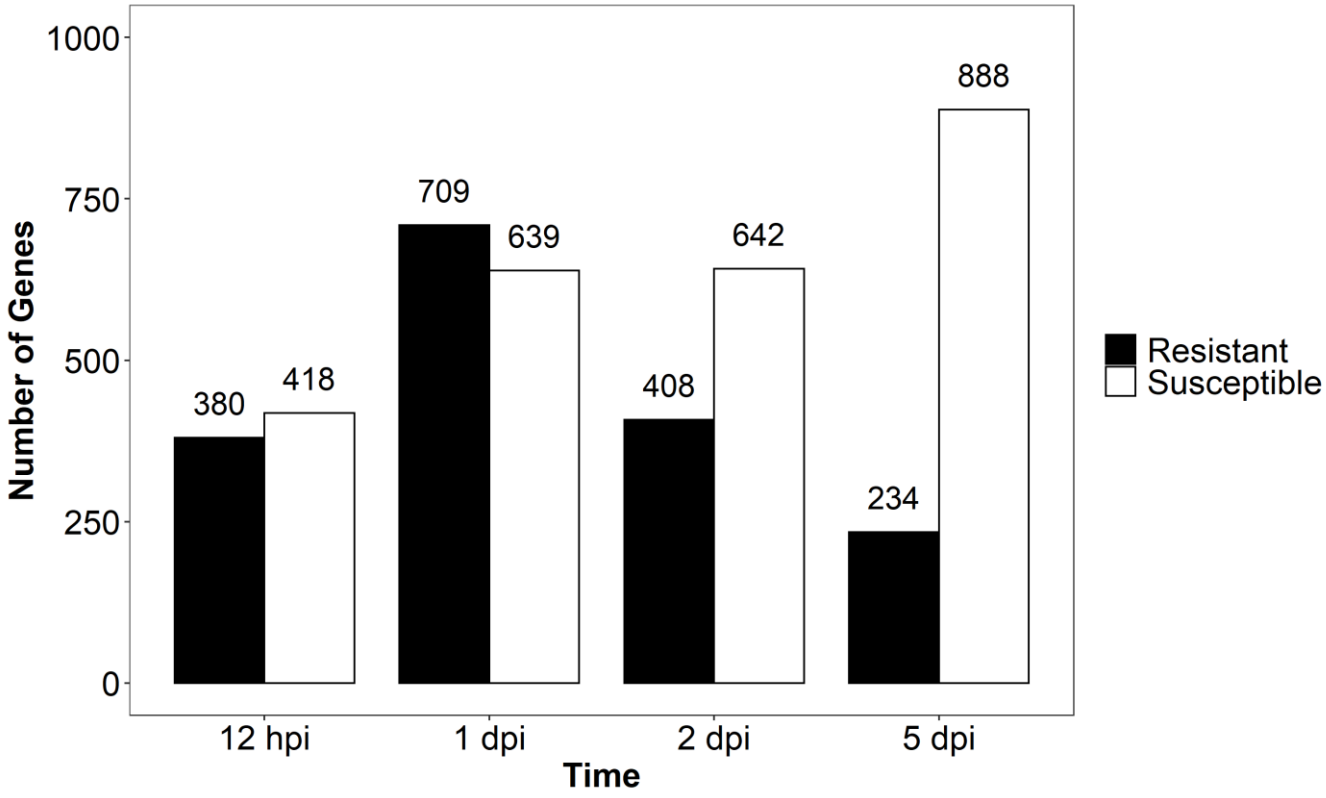

Figure 3.1. Number of total expressed *Austropuccinia psidii* genes over the time series. Black bars represent genes expressed in the resistant interaction while white bars represent genes expressed in the susceptible interaction. A total of 890 expressed genes were identified through RNA-seq analysis

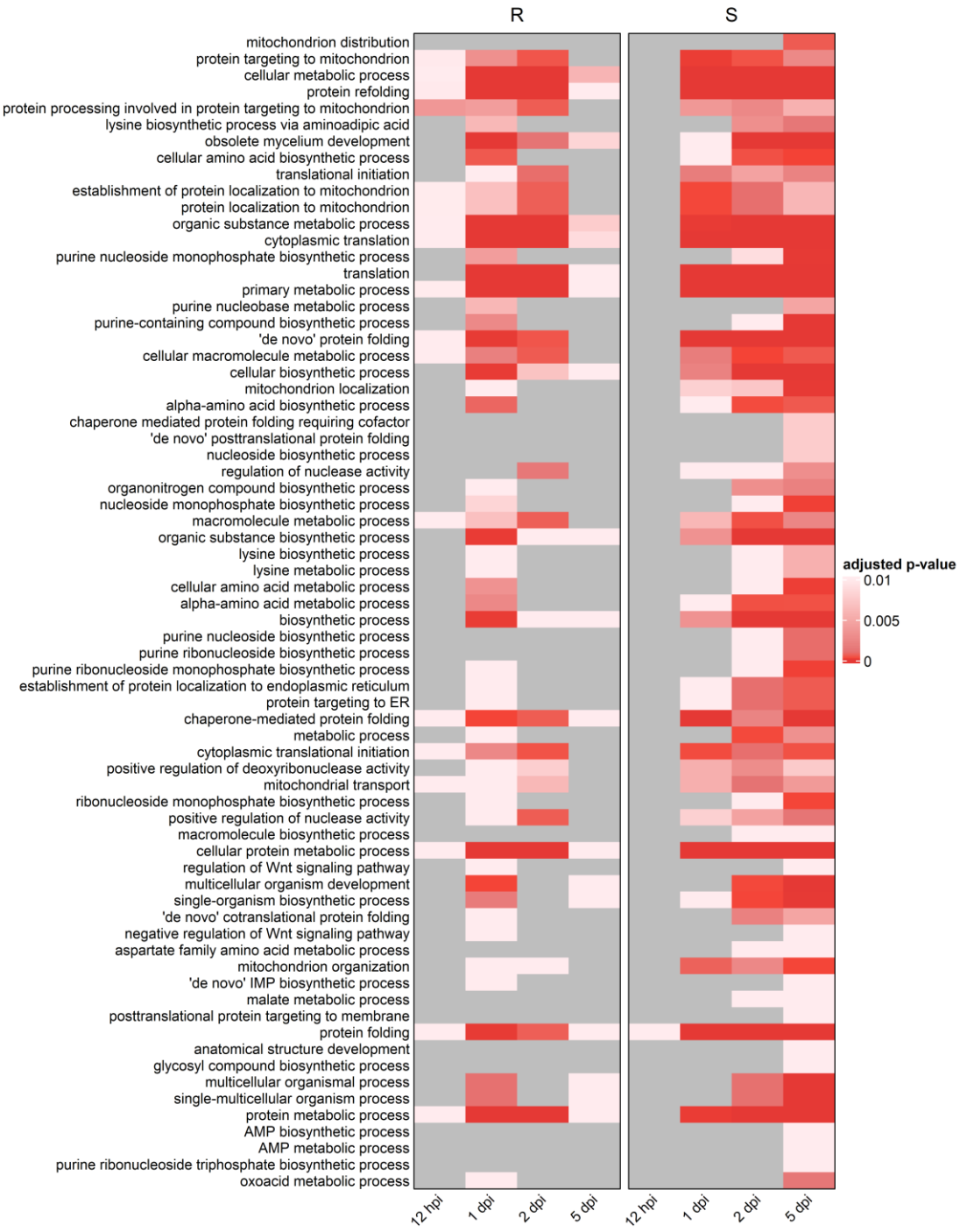

Figure 3.2. Over-represented gene ontologies (GO) in the biological processes (BP) category of total expressed *Austropuccinia psidii* genes in both the R- and S-interaction over the time series, where the colour scale represents the false discovery rate (FDR) adjusted p-value and grey represents absence of the term. GO analysis identified 188 over-represented BP terms, the heatmap represents the top 70 terms in relation to the lowest FDR value. R = resistant interaction; S = susceptible interaction; hpi = hours post inoculation; dpi = days post inoculation.

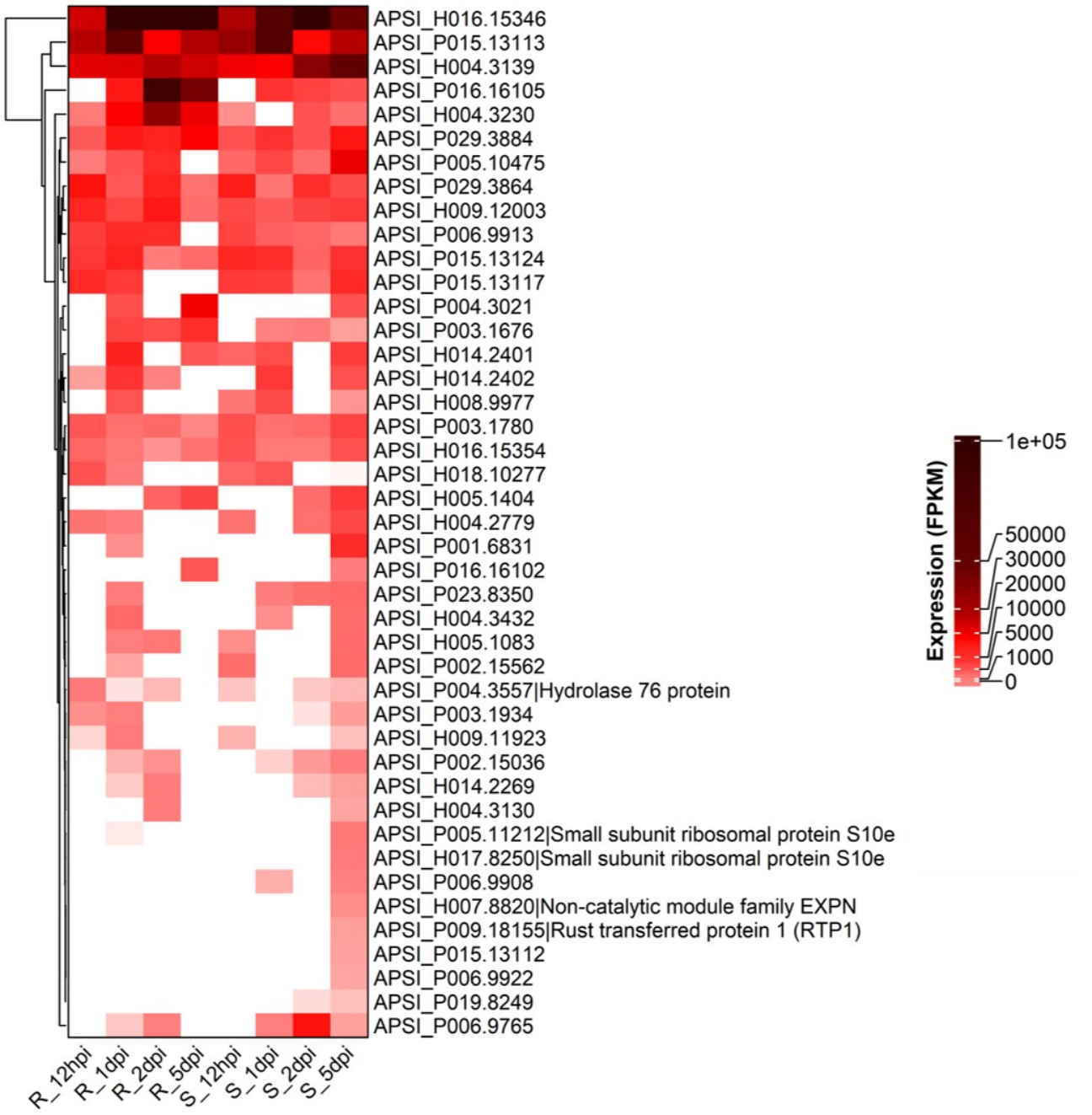

Figure 3.3. Heatmap showing the expression patterns of *Austropuccinia psidii* effectors genes in both the resistant and susceptible *Eucalyptus grandis* interactions over the time series. Genes with known annotations are labelled. The colour gradient represents the expression values of a measure of FPKM ranging from the minimum to maximum expression values, where white represents genes that are not expressed. FPKM = fragments per transcript million; hpi = hours post inoculation; dpi = days post inoculation; R = resistant interaction; S = susceptible interaction.

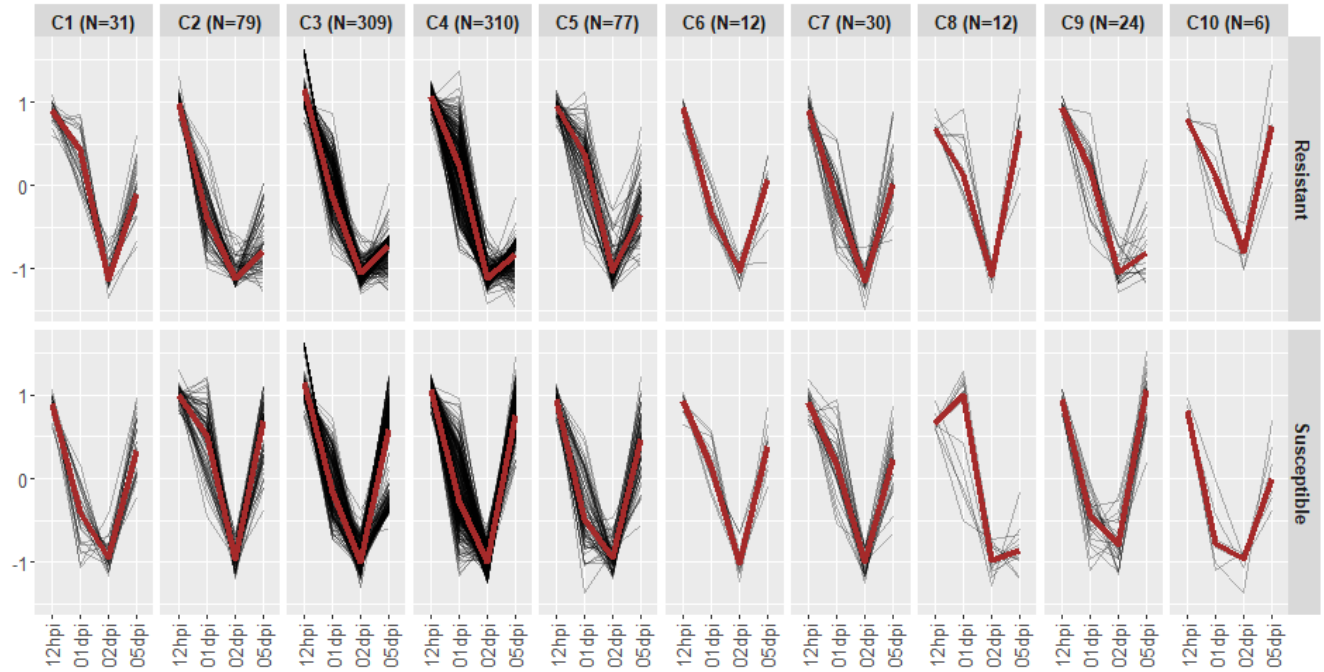

Figure 3.4. Line plots of each cluster across the time series in the resistant and susceptible samples. Each black line represents an expressed gene, and the red line represents the median expression in each cluster. The calculated Z-scores were used to plot the graphs. C = cluster; N = number of genes; hpi = hours post inoculation; dpi = days post inoculation; R = resistant interaction; S = susceptible interaction.

Chapter 4

Concluding Remarks

4.1 Introduction

Eucalyptus spp. are fast growing woody trees that are commonly cultivated for paper, pulp and timber and are valuable resources for bioenergy and biomaterials (Grattapaglia et al., 2012; Myburg et al., 2014). *Eucalyptus* spp. are highly susceptible to *Austropuccinia psidii,* a biotrophic rust pathogen that causes myrtle rust disease on numerous members of Myrtaceae (Carnegie and Pegg, 2018), with *E. grandis* responses ranging from highly susceptible to resistant (Junghans et al., 2003). *A. psidii* causes yellow pustules on leaves and shoots and severe infection leads to stunted growth and plant death (Fensham et al., 2020; Pegg et al., 2020). The pandemic biotype of *A. psidii* has been identified in various countries across the globe and poses severe threats to the natural and introduced ranges of Myrtaceae (Wingfield et al., 2015; Fensham et al., 2020).

In *E. grandis*, a major locus involved in resistance against *A. psidii* has been identified, termed *Puccinia psidii resistance 1* (*Ppr1*), named as per the previous taxonomy of *A. psidii* (Junghans et al., 2003). This locus was found to harbour numerous *R*-genes (Thumma et al., 2013). Despite this, trees harbouring this locus were found to be susceptible to infection, suggesting that the locus was failing at providing resistance (Graça et al., 2011). Since the initial discovery of this locus, various studies have emerged identifying additional loci conferring resistance against infection (Alves et al., 2012; Butler et al., 2016; Butler et al., 2019). This highlights the complex nature of resistance against *A. psidii.* Proteomic analysis of *A. psidii* spores in resistant *Eucalyptus* and susceptible guava revealed that proteins abundant in susceptible hosts included a plethora of fungal virulence factors such as malate dehydrogenase, heat shock proteins and CWDE (Quecine et al., 2016). Myrtle rust produces a concoction of potent enzymes that degrade the leaf waxy cuticle of *E. grandis,* facilitating the entry of the pathogen into the host plant (Xavier et al., 2015). Despite the significance of this pathogen, few studies have investigated the interaction between *A. psidii* and its hosts, partly due to its biotrophic nature. This emphasizes the importance of investigating these responses to gain further understanding of this complex pathosystem, with the aim to contribute to improved control of this devastating pathogen.

The improvement of next-generation technologies has vastly facilitated the investigations into the interactions between biotrophic rust pathogens and their associated hosts (Bakkeren and Szabo, 2020). Dual RNA-seq studies shed light on the *in planta* molecular dialogue that exists between host and pathogen, with this providing valuable insight into the mechanisms governing the complex interaction. These studies are aided by the *Eucalyptus grandis* v2.0 and *Austropuccinia psidii* reference genomes (Myburg et al., 2014; Tobias et al., 2021). There have been various studies that investigate the host

responses to infection through the use of high-throughput, robust RNA-seq analyses (Hsieh et al., 2018; Tobias et al., 2018; Santos et al., 2020; Hsieh et al., 2021; Sekiya et al., 2021). To date, there have been no studies investigating the transcriptional responses of *A. psidii* in its associated host plants, although it is expected that numerous studies will emerge with the recent release of the reference genome (Tobias et al., 2021). By investigating this pathosystem, we can identify novel host pathways and mechanisms involved in disease resistance, facilitating the manipulation of these mechanisms in molecular breeding to generate more tolerant plants. Additionally, by studying the transcriptomic changes in *A. psidii*, novel targets for disease control can be identified such as virulence and pathogenicity factors that will facilitate the mitigation of disease.

4.2 Summary of findings

The current study aimed to investigate the transcriptomic changes of *E. grandis* and *A. psidii* upon interaction through a robust dual RNA-seq approach. Seedlings of *E. grandis* were either inoculated with the pandemic biotype of *A. psidii* or mock-inoculated and monitored for their responses to infection (Swanepoel et al., 2021). Resistant and susceptible *E. grandis* were identified and leaf samples were obtained at four different time points (12-hpi, 1-, 2-, and 5-dpi) for mRNA-sequencing using Illumina HiSeq 2500. The RNA-seq data was used to investigate host responses in research chapter two and *in planta* pathogen responses in research chapter three.

The transcriptomic changes of *E. grandis* in response to *A. psidii* infection revealed that both resistant and susceptible hosts shared similar responses. Analyses revealed that there was significant up-regulation of defence-related terms in both hosts, with little down-regulation of defence terms. This suggests that both hosts are able to mount a defence response against infection and the differences observed in the phenotypes is attributed to the efficiency in these responses. Responses appeared to begin earlier in the S-interaction at 12-hpi, with up-regulation of terms such as hydrogen peroxide metabolism and phytohormone-related terms. These responses were absent in the R-interaction at 12-hpi. This may be due to insufficient preformed barriers of defence in the S-interaction that facilitated the rapid penetration of pathogen structures into the host plant. A previous study has found that hyphae were observed in susceptible *E. grandis* as early as 12-hpi, while similar structures were only observed in resistant hosts at 18-hpi (Xavier et al., 2001). Responses begin rapidly increasing in the R-interaction at 1- and 2-dpi, with similar responses absent in the S-interaction. These responses are sustained throughout the time series, while absence in the S-interaction suggests that an uncoordinated and dampened response results in disease. This highlights the importance of timing and coordination of responses.

Brassinosteroids have been implicated in disease resistance against various biotrophic pathogens (Yu et al., 2018). In this study, BR signalling has been identified in resistant hosts at 2-dpi, while similar responses are absent in the S-interaction (Swanepoel et al., 2021). This suggests that BR signalling is involved in the defence responses against myrtle rust. Various genes involved in BR signalling were identified, including *BAK1* genes. This protein is involved in BR signal transduction and has been found to be involved in PTI responses against pathogenic organisms. The expression of this gene was significantly more DE in the R-interaction, suggesting its involvement in the interaction with *A. psidii*. Analysis of the genes underlying the resistance loci (*Ppr1-Ppr5*) revealed two and four BR signalling genes within *Ppr3* and *Ppr5*, respectively. This suggests that the BR signalling contributes to the resistance mechanisms underlying these resistance loci.

Further analyses of the resistance loci revealed numerous *R*-genes within the loci, and studies on their expression showed that various are DE in the presence of *A. psidii*. DE in the R-interaction was greater between 1- and 2-dpi while similar expression of these genes was only observed at 5-dpi in the S-interaction, suggesting that DE earlier in the interaction contributes to successful prevention of disease. Analysis between mock-inoculated resistant and susceptible *E. grandis* revealed differences in the constitutive expression of *R*-genes underlying these loci, suggesting that basal expression of these genes contributes to host responses before infection with *A. psidii* (Swanepoel et al., 2021). This is further supported by Santos et al. (2020) where the authors found constitutive expression of R proteins putatively contributing to host resistance.

The *in planta* responses of *A. psidii* in resistant and susceptible *E. grandis* is explored in chapter three. In this chapter, we found that despite the drastically different disease symptoms in resistant and susceptible hosts, the responses of the pathogen were similar. Corroborating the responses of the host, where expression of genes began earlier in the S-interaction (Swanepoel et al., 2021), there were more pathogen genes expressed in the S-interaction at 12-hpi. Resistant *E. grandis* rapidly increased in response at 1-dpi, which correlates to the increase in pathogen gene expression at this time point. Resistant defence responses seemed to be sufficient enough to dampen pathogen gene expression by 2-dpi, where there was a dramatic decrease in expression of genes. At 5-dpi in the S-interaction, the most amount of pathogen expressed genes were found, suggesting that proliferation of *A. psidii* was successful at this time point and correlates to the increased expression of host specific genes, which may be an over-compensation from susceptible hosts to combat pathogen infection.

We identified very few DEGs, suggesting that expression of these genes in resistant and susceptible hosts was similar. Performing enrichment on total expressed genes, we found numerous pathways that

were present in the S-interaction and absent in the R-interaction. Malate dehydrogenase activity enriched in the S-interaction at 2- and 5-dpi. Furthermore, oxoacid metabolism was enriched in the S-interaction at 5-dpi. Malate dehydrogenase is an enzyme that converts precursor molecules to oxalic acid, with oxalic acid forming part of oxoacids. Oxalic acid is known to reduce plant ROS (Cessna et al., 2000), to reduce plant defence responses and facilitate disease symptoms. This correlates with the responses observed in the S-interaction, where oxidative burst responses appeared to be inconsistently regulated. The interruption of ROS in susceptible hosts may contribute to disease susceptibility.

Analyses of the candidate *A. psidii* effectors revealed that numerous effector genes are expressed in the interactions in resistant and susceptible *E. grandis*. This revealed the expression of a rust specific gene in the S-interaction at 5-dpi, a gene that is absent in the R-interaction. This protein is involved in inhibiting the movement of chloroplasts and the nucleus within the host cell, preventing vital functioning of the host cell (Kemen et al., 2013), suggesting its requirement for pathogenicity of *A. psidii*. Additionally, a candidate effector hydrolase 76 protein family gene was identified in both the R- and S-interaction, suggesting that CWDE are crucial pathogenicity factors in both resistant and susceptible hosts.

4.3 Contributions to current knowledge

At the beginning of this study, there were few studies that investigated the transcriptomic responses of Myrtaceae species in response to *A. psidii* (Hsieh et al., 2018; Tobias et al., 2018). Recent studies have emerged that investigate the host responses against *A. psidii* (Santos et al., 2020) but no studies have emerged that investigate host responses over a time series upon infection with *A. psidii*. In the present study, a comprehensive time series reveals how the responses of *E. grandis* changes over the course of infection, revealing that timing of responses is important in the interaction. We show that despite the resistant hosts mounting a defence response after the susceptible hosts, coordination and efficiency in responses in the resistant hosts are more important, as susceptible hosts are unable to sustain defence responses and prevent pathogen proliferation. Furthermore, we show that susceptible hosts mount extreme responses at 5-dpi, and these responses may be an over-compensation due to the increase in disease severity at this time point. We identified brassinosteroid signalling in the R-interaction at 2-dpi, with this phytohormone signalling process absent in the S-interaction across the time series. This is the first report of this phytohormone in the host-*A. psidii* pathosystem. Future studies need to investigate how this phytohormone contributes to host defence. Finally, we identified a plethora of resistance genes that are significantly differentially expressed both at the constitutive and induced

levels. These genes are tagged as contributors to host defence and further studies are required to determine how they are involved in the interaction.

To date, there are no studies that investigate the transcriptomic responses of *A. psidii* in *E. grandis* and other Myrtaceae species, however new studies are expected to emerge due to the release of the *Austropuccinia psidii* reference genome (Tobias et al., 2021). We identified pathways involved in pathogenicity and virulence, corroborated by previous studies such as Quecine et al. (2016), such as the involvement of malate dehydrogenase and oxalic acid in host susceptibility. Additionally, we identified a rust transferred protein in the S-interaction at 5-dpi. This is the first report of the involvement of this protein in the *A. psidii* pathosystem. This study has identified pathways and genes that potentially contribute to its success in susceptible hosts.

The results of this study provide valuable insights into the complex molecular dialogue that exists between *E. grandis* and *A. psidii*. Through this, we have identified novel pathways putatively involved in the resistance against myrtle rust. This includes a range of resistance genes and novel phytohormone pathways. These findings will significantly contribute to targeted selective breeding programmes and genetic engineering of resistant plants to mitigate the devastating effects of the pathogen. Moreover, cell wall degrading enzymes, a plethora of effector proteins, and oxalic acid putatively contribute to the molecular dialogue between myrtle rust and *E. grandis.* The identified effector genes can be the target for future functional studies that aims to characterise the *in planta* functions, as this will significantly contribute to novel disease control strategies. Overall, we have demonstrated the molecular dialogue between host and pathogen over a time series, with this being the first study of its kind. The knowledge gained from this study will drive future studies that further unravel the mechanisms underlying these interactions.

4.4 Hypothesised model of the molecular dialogue

We hypothesise the model in Figure 4.1 to explain the molecular dialogue between *A. psidii* and *E. grandis*. The dialogue between host and pathogen triggers significant changes within the transcriptome of both resistant and susceptible hosts. In susceptible *E. grandis*, we hypothesise that the effector proteins released during the interaction can go undetected by the plant *R*-genes and thus, downstream defence responses including HR and ROS are inhibited. The accumulation of RTP1 near the haustoria in the extra-haustoria matrix results in accumulation of chloroplasts and the nucleus near the haustoria. This results in reduced functioning of the chloroplasts, which may affect defence responses such as production of ROS and HR. HR and ROS are also affected in the presence of oxalic acid. In

combination, susceptible hosts may not be able to activate HR. This is observed in Swanepoel et al. (2021), in which susceptible hosts showed delayed HR, only during late stages of infection at 5-dpi. Our results showed that phytohormones involved in the response included SA, JA, ET, ABA and BR. In susceptible hosts, the production of oxalic acid by *A. psidii* might be affecting the production of JA, ET and ABA which may influence the SA-related defence responses. In turn, this may affect the production of BR within susceptible hosts, accounting for its absence in our study. This may contribute to reduced activation of downstream defence responses that result in increased disease symptoms.

4.5 Limitations and future studies

Despite the robust RNA-seq analysis, and the stringency with which the data was analysed, there are limited further lines of evidence to validate the responses we are observing. Future studies need to focus on brassinosteroid signalling in both resistant and susceptible hosts to confirm that this phytohormone is involved in resistance. This can be through direct measurements of BR levels upon infection, or inhibition of BR biosynthesis and signalling in resistant hosts while monitoring the resulting responses upon infection.

We identified little pathogen data, with only a total of 890 genes identified in the interaction. The expression of these genes provides valuable insights of the pathogen interactions. However, there are far more genes involved in this interaction that we are not finding in the present study, so future studies need to investigate targeted areas upon infection, such as harvesting tissues directly surrounding the urediniospores pustules. This will ensure we identify more pathogen genes to gain an understanding of the whole picture. This is not always possible with studies on rust pathogens, as pustules typically develop at 10-dpi. This means that a study focusing on the pustules will only obtain information of late stages of infection and not the responses occurring during early colonisation. Despite this, information on gene expression ranging from early infection, colonisation and proliferation will provide comprehensive, valuable information on the responses of *A. psidii*.

Finally, we were unable to perform a comprehensive network analysis including both the host and pathogen data as the pathogen data did not fit the parameters for a weighted gene co-expression network analysis (WGCNA). Therefore, we could not investigate the relationship between expressed host and pathogen genes, and only a hierarchical clustering could be performed on pathogen data. Hierarchical clustering results are informative, but it does not show the relationship between host and pathogen. Future studies will need to isolate fungal structures for RNA extraction to obtain more pathogen data for the analysis, such as haustoria or pustules.

4.6 References

Alves, A.A., Rosado, C.C.G., Faria, D.A., Da Silva Guimarães, L.M., Lau, D., Brommonschenkel, S.H., et al. (2012). Genetic mapping provides evidence for the role of additive and non-additive QTLs in the response of inter-specific hybrids of *Eucalyptus* to *Puccinia psidii* rust infection. *Euphytica* 183**,** 27-38. doi: 10.1007/s10681-011-0455-5.

Bakkeren, G., and Szabo, L.J. (2020). Progress on molecular genetics and manipulation of rust fungi. *Phytopathology* 110**,** 532-543. doi: 10.1094/PHYTO-07-19-0228-IA.

Butler, J.B., Freeman, J.S., Vaillancourt, R.E., Potts, B.M., Glen, M., Lee, D.J., et al. (2016). Evidence for different QTL underlying the immune and hypersensitive responses of *Eucalyptus globulus* to the rust pathogen *Puccinia psidii*. *Tree Genetics & Genomes* 12**,** 39. doi: 10.1007/s11295-016-0987-x.

Butler, J.B., Potts, B.M., Vaillancourt, R.E., Lee, D.J., Pegg, G.S., and Freeman, J.S. (2019). Independent QTL underlie resistance to the native pathogen *Quambalaria pitereka* and the exotic pathogen *Austropuccinia psidii* in *Corymbia*. *Tree Genetics & Genomes* 15**,** 72. doi: 10.1007/s11295-019-1378-x.

Carnegie, A.J., and Pegg, G.S. (2018). Lessons from the incursion of myrtle rust in Australia. *Annual Review of Phytopathology* 56**,** 457-478. doi: 10.1146/annurev-phyto-080516-035256.

Cessna, S.G., Sears, V.E., Dickman, M.B., and Low, P.S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell* 12**,** 2191-2199. doi: 10.1105/tpc.12.11.2191.

Fensham, R.J., Carnegie, A.J., Laffineur, B., Makinson, R.O., Pegg, G.S., and Wills, J. (2020). Imminent extinction of Australian Myrtaceae by fungal disease. *Trends in Ecology & Evolution* 35**,** 7. doi: 10.1016/j.tree.2020.03.012.

Graça, R.N., Aun, C.P., Guimarães, L.M.S., Rodrigues, B.V.A., Zauza, E.a.V., and Alfenas, A.C. (2011). A new race of *Puccinia psidii* defeats rust resistance in eucalypt. *Australasian Plant Pathology* 40**,** 442-447. doi: 10.1007/s13313-011-0056-8.

Grattapaglia, D., Vaillancourt, R.E., Shepherd, M., Thumma, B.R., Foley, W., Külheim, C., et al. (2012). Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. *Tree Genetics & Genomes* 8**,** 463-508. doi: 10.1007/s11295-012-0491-x.

Hsieh, J.-F., Chuah, A., Patel, H.R., Sandhu, K.S., Foley, W.J., and KüLheim, C. (2018). Transcriptome profiling of *Melaleuca quinquenervia* challenged by myrtle rust reveals differences in defense responses among resistant individuals. *Phytopathology* 108**,** 495-509. doi: 10.1094/PHYTO-09-17-0307-R.

Hsieh, J.-F., Krause, S.T., Kainer, D., Degenhardt, J., Foley, W.J., and Külheim, C. (2021). Characterization of terpene biosynthesis in *Melaleuca quinquenervia* and ecological consequences of terpene accumulation during myrtle rust infection. *Plant-Environment Interactions* 2**,** 177-193. doi: 10.1002/pei3.10056.

Junghans, D.T., Alfenas, A.C., Brommonschenkel, S.H., Oda, S., Mello, E.J., and Grattapaglia, D. (2003). Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. *Theoretical and Applied Genetics* 108**,** 175-180. doi: 10.1007/s00122-003-1415-9.

Kemen, E., Kemen, A., Ehlers, A., Voegele, R., and Mendgen, K. (2013). A novel structural effector from rust fungi is capable of fibril formation. *Plant Journal* 75**,** 767-780. doi: 10.1111/tpj.12237.

Myburg, A.A., Grattapaglia, D., Tuskan, G.A., Hellsten, U., Hayes, R.D., Grimwood, J., et al. (2014). The genome of *Eucalyptus grandis*. *Nature* 510**,** 356. doi: 10.1038/nature13308.

Pegg, G.S., Entwistle, P., Giblin, F.R., and Carnegie, A.J. (2020). Fire and rust – the impact of *Austropuccinia psidii* (myrtle rust) on regeneration of Myrtaceae in coastal heath following wildfire. *Southern Forests: A Journal of Forest Science* 82**,** 280-291. doi: 10.2989/20702620.2020.1819154.

Quecine, M.C., Leite, T.F., Bini, A.P., Regiani, T., Franceschini, L.M., Budzinski, I.G.F., et al. (2016). Label-free quantitative proteomic analysis of *Puccinia psidii* uredospores reveals differences of fungal populations infecting *Eucalyptus* and guava. *PLoS One* 11**,** e0145343. doi: 10.1371/journal.pone.0145343.

Santos, S.A., Vidigal, P.M.P., GuimarãEs, L.M.S., Mafia, R.G., Templeton, M.D., and Alfenas, A.C. (2020). Transcriptome analysis of *Eucalyptus grandis* genotypes reveals constitutive overexpression of genes related to rust (*Austropuccinia psidii*) resistance. *Plant Molecular Biology*. doi: 10.1007/s11103-020-01030-x.

Sekiya, A., Marques, F.G., Leite, T.F., Cataldi, T.R., De Moraes, F.E., Pinheiro, A.L.M., et al. (2021). Network analysis combining proteomics and metabolomics reveals new insights into early responses of *Eucalyptus grandis* during rust infection. *Frontiers in Plant Science* 11. doi: 10.3389/fpls.2020.604849.

Swanepoel, S., Oates, C.N., Shuey, L.S., Pegg, G.S., and Naidoo, S. (2021). Transcriptome analysis of *Eucalyptus grandis* implicates brassinosteroid signaling in defense against myrtle rust (*Austropuccinia psidii*). *Frontiers in Forests and Global Change* 4**,** 778611. doi: 10.3389/ffgc.2021.778611.

Thumma, B., Pegg, G.S., Warburton, P., Brawner, J.T., Macdonell, P., Yang, X., et al. (2013). Molecular tagging of rust resistance genes in eucalypts. 1-15.

Tobias, P.A., Guest, D.I., Külheim, C., and Park, R.F. (2018). De novo transcriptome study identifies candidate genes involved in resistance to *Austropuccinia psidii* (myrtle rust) in *Syzygium luehmannii* (riberry). *Phytopathology* 108**,** 627-640. doi: 10.1094/PHYTO-09-17-0298-R.

Tobias, P.A., Schwessinger, B., Deng, C.H., Wu, C., Dong, C., Sperschneider, J., et al. (2021). *Austropuccinia psidii,* causing myrtle rust, has a gigabase-sized genome shaped by transposable elements. *G3 Genes|Genomes|Genetics* 11**,** jkaa015. doi: 10.1093/g3journal/jkaa015.

Wingfield, M.J., Brockerhoff, E.G., Wingfield, B.D., and Slippers, B. (2015). Planted forest health: The need for a global strategy. *Science* 349**,** 832-836. doi: 10.1126/science.aac6674.

Xavier, A.A., Alfenas, A.C., Matsuoka, K., and Hodges, C.S. (2001). Infection of resistant and susceptible *Eucalyptus grandis* genotypes by urediniospores of *Puccinia psidii*. *Australasian Plant Pathology* 30**,** 277-281. doi: 10.1071/ap01038.

Xavier, A.A., Da Silva, A.C., Da Silva Guimarães, L.M., Matsuoka, K., Hodges, C.S., and Alfenas, A.C. (2015). Infection process of *Puccinia psidii* in *Eucalyptus grandis* leaves of different ages. *Tropical Plant Pathology* 40**,** 318-325. doi: 10.1007/s40858-015-0043-7.

Yu, M.-H., Zhao, Z.-Z., and He, J.-X. (2018). Brassinosteroid signaling in plant-microbe interactions. *International Journal of Molecular Sciences* 19**,** 4091. doi: 10.3390/ijms19124091.

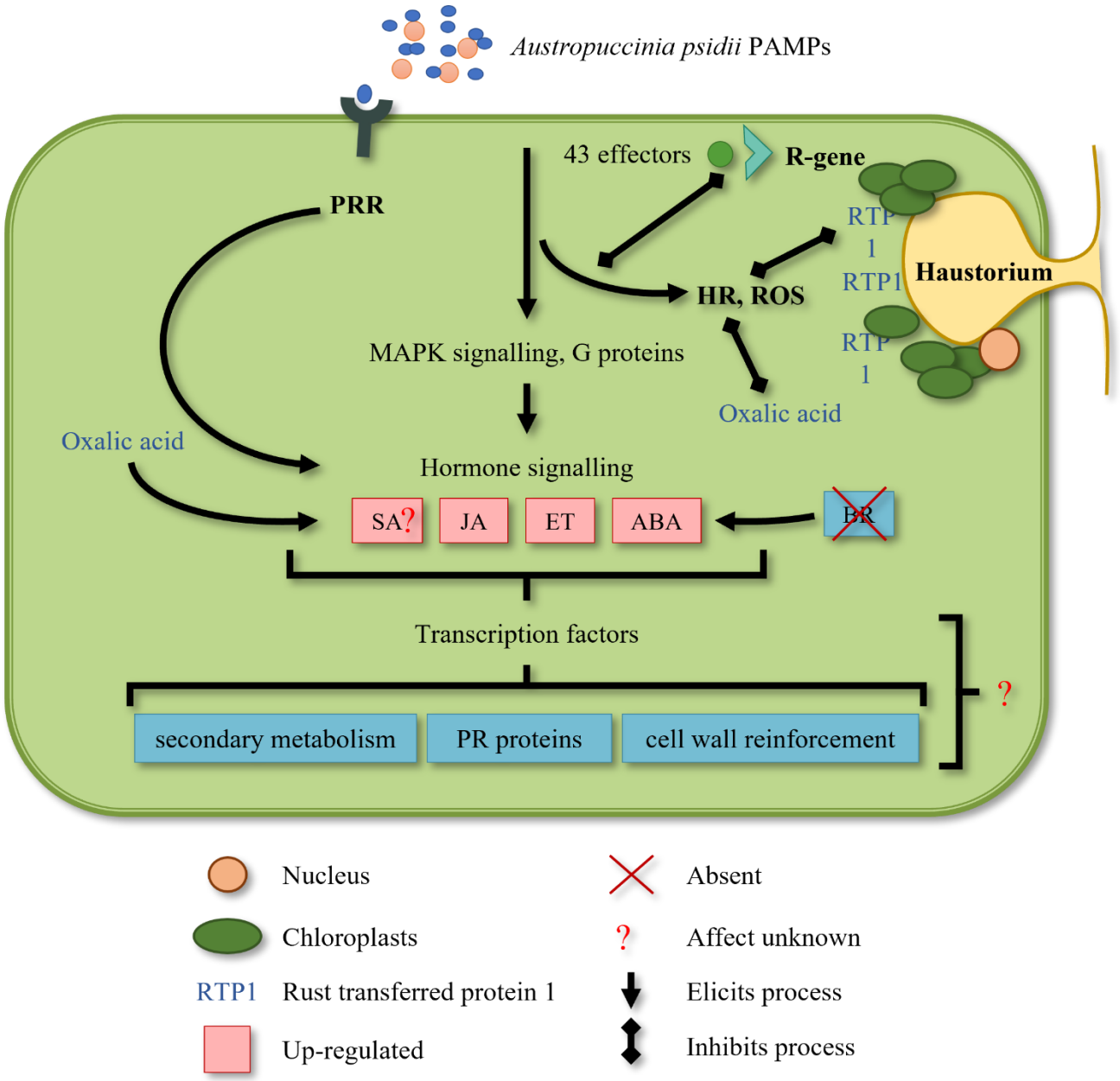

Figure 4.1. Hypothesised model of the interaction between *Eucalyptus grandis* and *Austropuccinia psidii.* ABA = abscisic acid; BR = brassinosteroids; ET = ethylene; HR = hypersensitive response; JA = jasmonic acid; PR = pathogenesis-related protein; PRR = pathogen recognition receptor; *R*-gene = resistance gene; ROS = reactive oxygen species; RTP1 = rust transferred protein 1; SA = salicylic acid.

Supplementary materials

The datasets generated for this study can be found through the National Centre for Biotechnology Information (NCBI) Short Read Archive (SRA) databases under the following accession, PRJNA763498.

The supplementary figures and tables can be found at:

https://drive.google.com/drive/u/1/folders/0AMtCMNvIcHX8Uk9PVA

Chapter 2

Supplementary Table 2.1: Tables showing the number of total gene and their descriptions underlying *Puccinia psidii resistance* (*Ppr*) loci.

Supplementary Table 2.2: Tables showing differentially expressed genes underlying *Puccinia psidii resistance* (*Ppr*) loci in R, S and S-C vs R-C.

Supplementary Table 2.3: Tables showing differentially expressed resistance (R) genes underlying *Puccinia psidii resistance* (*Ppr*) loci in resistant, susceptible and resistant, control vs. susceptible, control.

Supplementary Table 2.4: Table showing the number and identity of brassinosteroid mediated signalling genes underlying *Puccinia psidii resistance* (*Ppr*) loci.

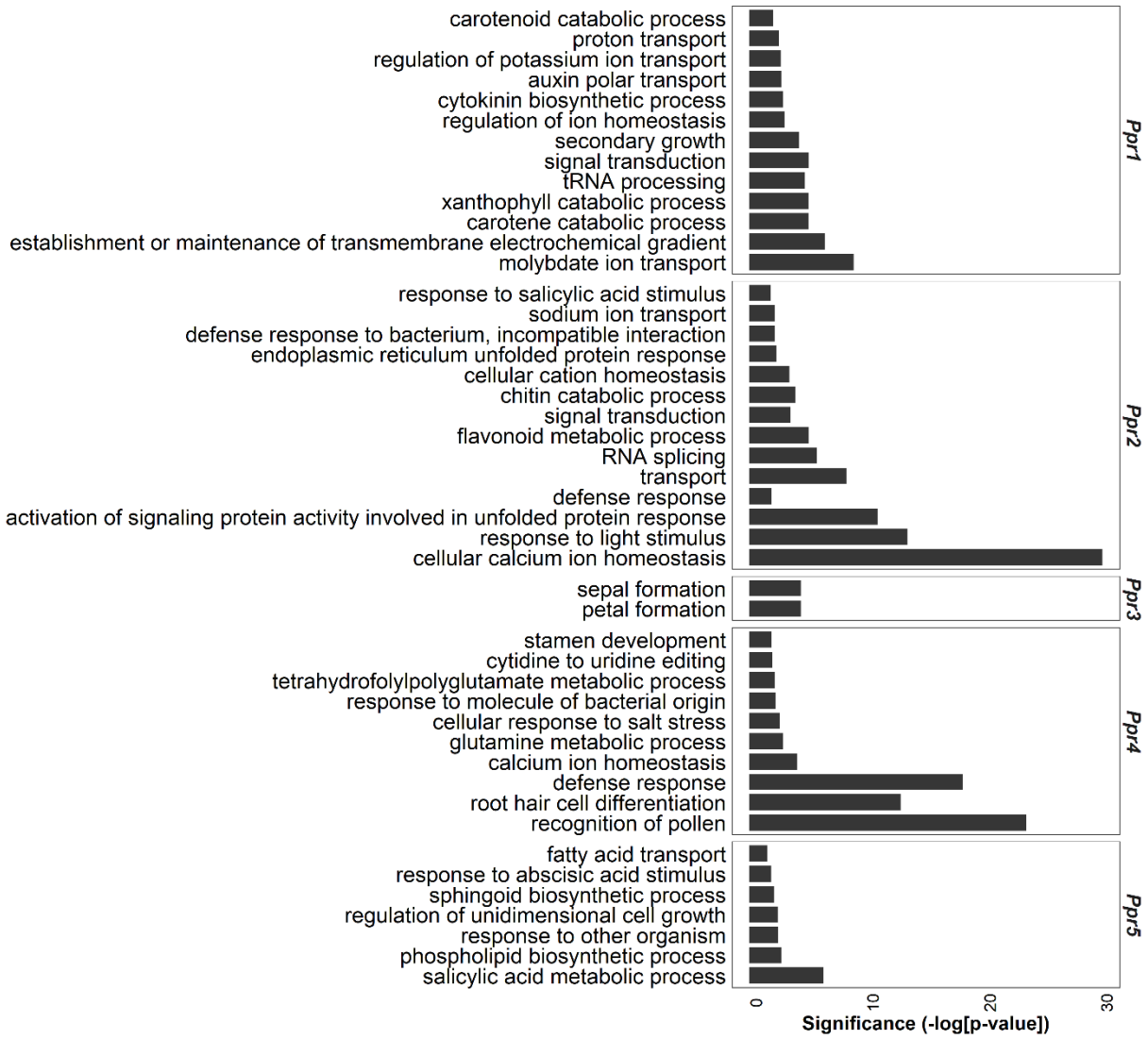

Figure S2.1. The gene ontology (GO) biological processes functional characterisation of genes underlying the five resistance loci in *Eucalyptus* (*Ppr1-5*), showing the significantly enriched terms in each locus.

Chapter 3

Supplementary Table 3.1: The mapping statistics of the dual RNA-seq of *Eucalyptus grandis* and *Austropuccinia psidii.*

Supplementary Table 3.2: The top 100 most highly expressed *Austropuccinia psidii* genes in the resistant interaction, as a measure of FPKM values.

Supplementary Table 3.3: The top 100 most highly expressed *Austropuccinia psidii* genes in the susceptible interaction, as a measure of FPKM values.

Supplementary Table 3.4: Over-represented KEGG terms of total expressed *Austropuccinia psidii* genes representing FDR values.

Supplementary Table 3.5: Differentially expressed gene list at 5-dpi.

Supplementary Table 3.6: Over-represented gene ontologies (GO) in the biological processes (BP) category of total expressed *Austropuccinia psidii* genes representing FDR values.

Supplementary Table 3.7: The expressed candidate *Austropuccinia psidii* effectors representing FPKM values.

Supplementary Table 3.8: The top ten BLAST results for expressed candidate effectors.

Supplementary Table 3.9: The clusters of total expressed *Austropuccinia psidii* genes identified through hierarchical clustering.

Supplementary Table 3.10: The GO enrichment on clusters of total expressed *Austropuccinia psidii* genes identified through hierarchical clustering.

Supplementary Table 3.11: The CRB BLAST results with target proteins identified in a previous proteome study.

Supplementary Table 3.12: The expressed genes from the CRB BLAST results.

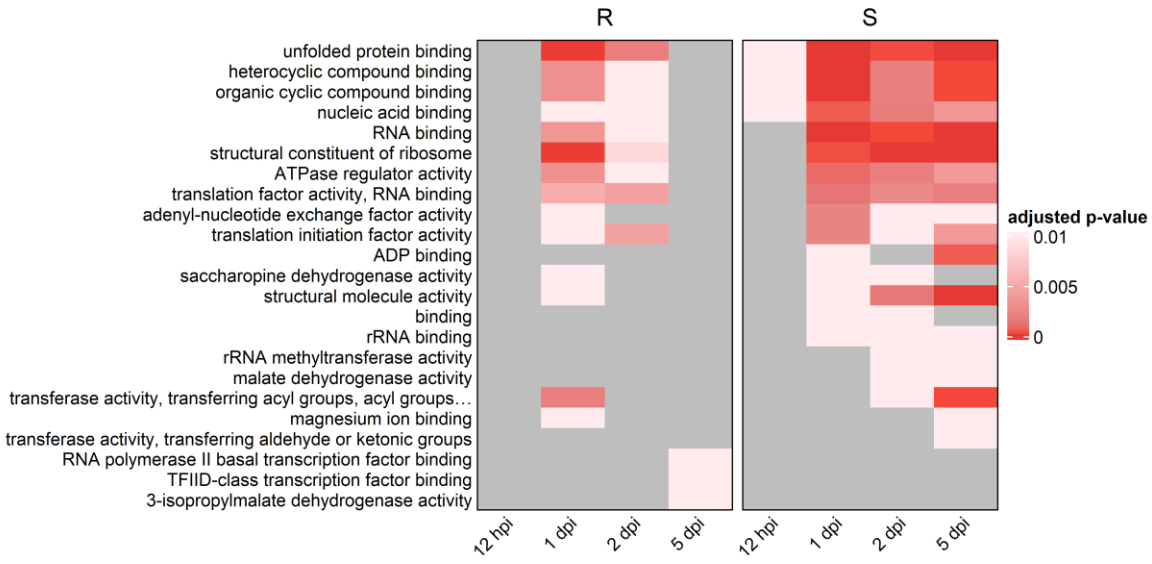

Figure S3.1. Over-represented gene ontologies (GO) in the molecular function (MF) category. The heatmap shows enriched terms of the total expressed *Austropuccinia psidii* genes in both the R- and S-interaction over the time series, where the colour scale represents the false discovery rate (FDR) adjusted p-value and grey represents the absence of the term. GO analysis identified 23 over-represented MF terms. R = resistant interaction; S = susceptible interaction; hpi = hours post inoculation; dpi = days post inoculation.

Figure S3.2. Over-represented gene ontologies (GO) in the cellular component (CC) category. The heatmap shows enriched terms of the total expressed *Austropuccinia psidii* genes in both the R- and S-interaction over the time series, where the colour scale represents the false discovery rate (FDR) adjusted p-value and grey represents the absence of the term. GO analysis identified 77 over-represented CC terms. R = resistant interaction; S = susceptible interaction; hpi = hours post inoculation; dpi = days post inoculation.

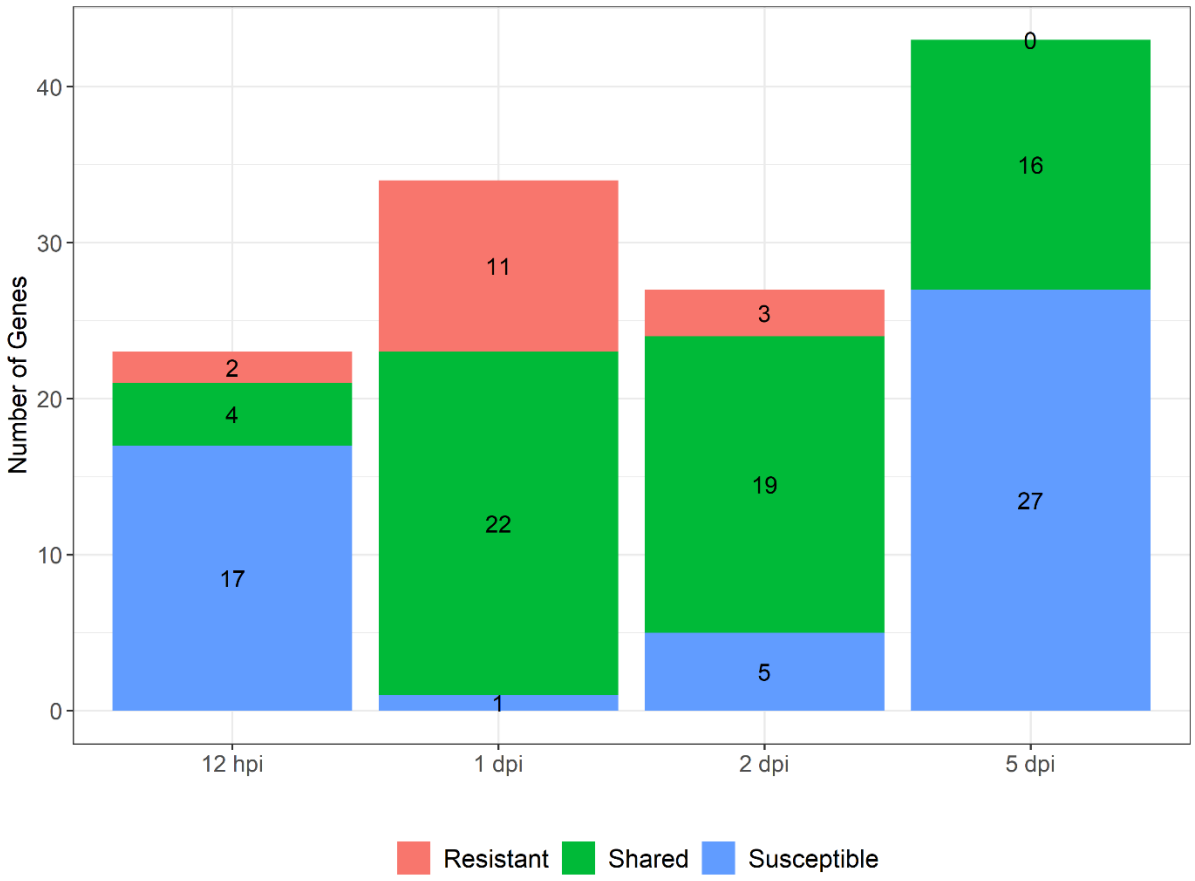

Figure S3.3. The number of expressed *Austropuccinia psidii* candidate effectors. Red represents the genes expressed in the R-interaction, green represents expressed genes between the R- and S-interaction, and blue represents effector genes expressed in the S-interaction. hpi = hours post inoculation; dpi = days post inoculation.

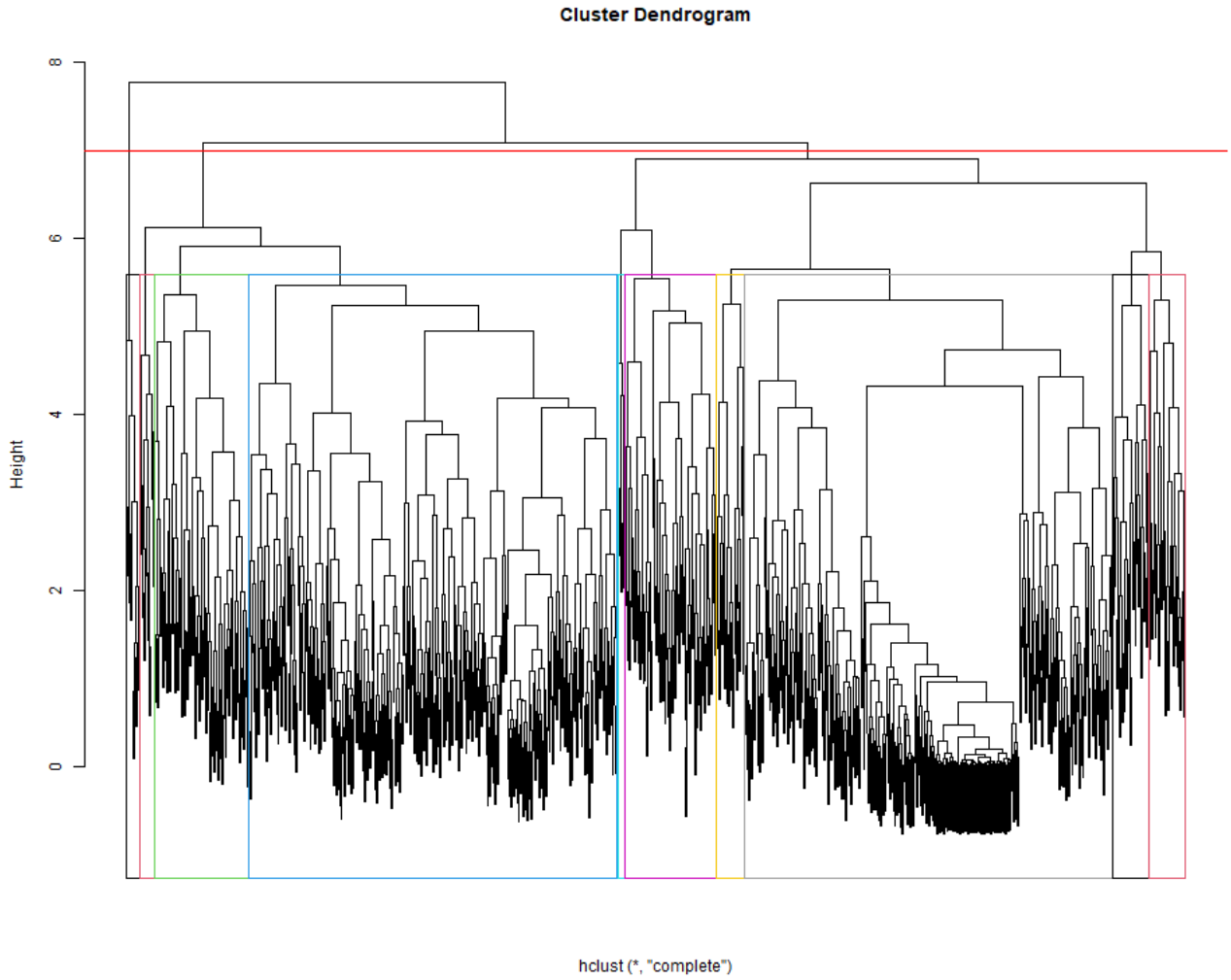

Figure S3.4. The cluster dendrogram of expressed *Austropuccinia psidii* genes in inoculated samples. The number of clusters selected from this dendrogram was K = 10, shown by the colour blocks. The red line indicates where the dendrogram would have been cut if K = 3, generating three clusters.

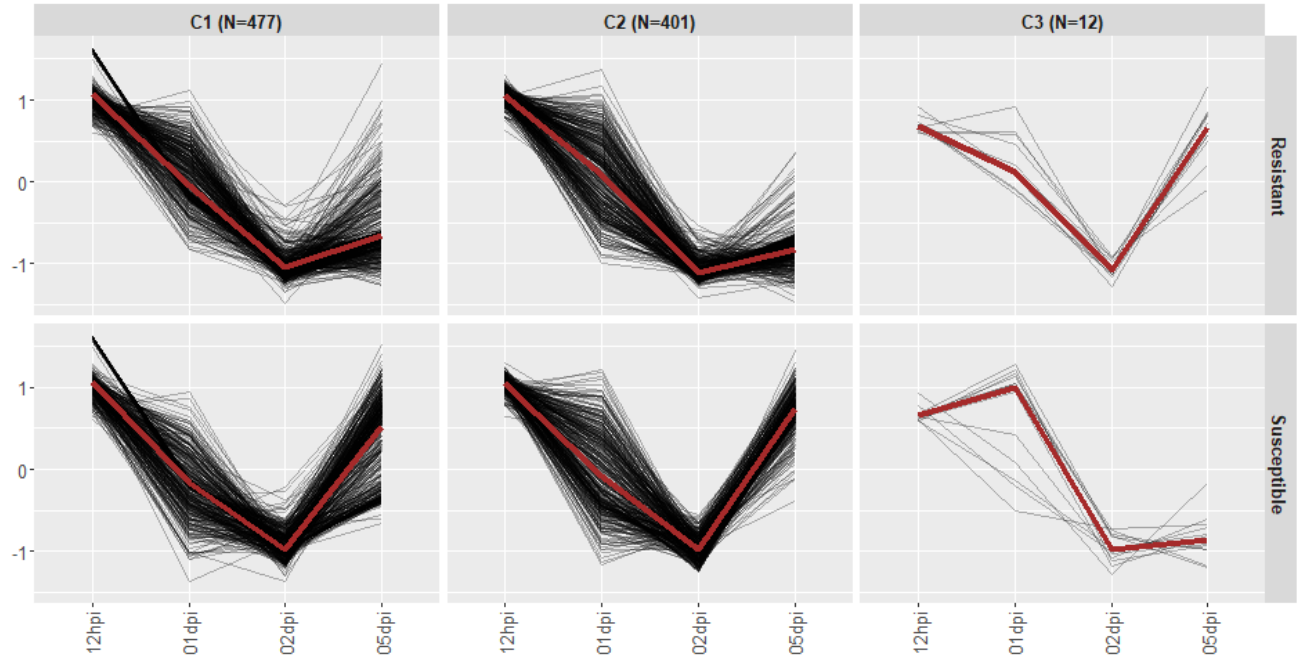

Figure S3.5. Line plots of each cluster across the time series in the resistant and susceptible samples where K = 3. Each black line represents an expressed gene, and the red line represents the median expression in each cluster. The calculated Z-scores were used to plot the graphs. C = cluster; N = number of genes; hpi = hours post inoculation; dpi = days post inoculation; R = resistant interaction; S = susceptible interaction.

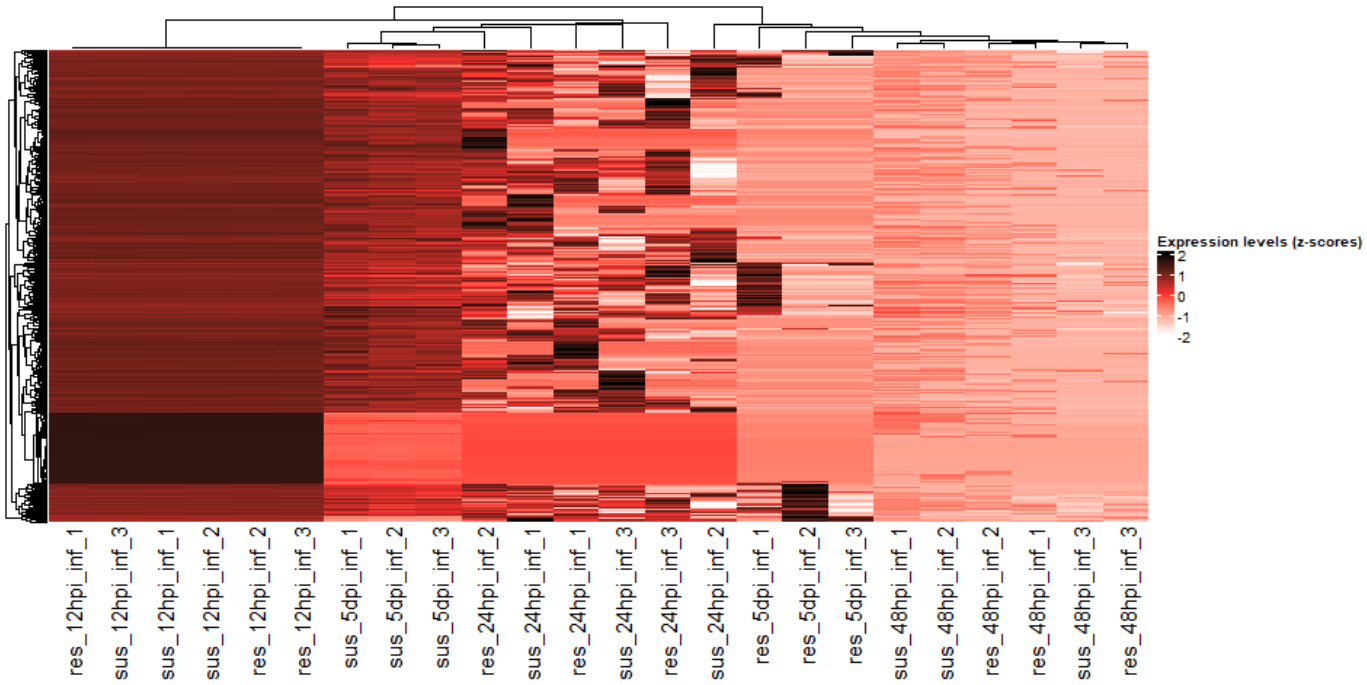

Figure S3.6: Hierarchical clustering analysis of expressed *Austropuccinia psidii* genes showing a heatmap of expression across the time series in both the resistant and susceptible interactions. The VST values were scaled by Z-scores. The colour gradient represents the relative expression of the genes, where black represents greater expression and white represents lower expression.