

# Analysis of Plant and Fungal Transcripts from Resistant and Susceptible Phenotypes of *Leptospermum scoparium* Challenged by *Austropuccinia psidii*

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## Abstract

*Austropuccinia psidii* is the causal pathogen of myrtle rust disease of Myrtaceae. To gain understanding of the initial infection process, gene expression in germinating *A. psidii* urediniospores and in *Leptospermum scoparium*-inoculated leaves were investigated via analyses of RNA sequencing samples taken 24 and 48 h postinoculation (hpi). Principal component analyses of transformed transcript count data revealed differential gene expression between the uninoculated *L. scoparium* control plants that correlated with the three plant leaf resistance phenotypes (immunity, hypersensitive response, and susceptibility). Gene expression in the immune resistant plants did not significantly change in response to fungal inoculation, whereas susceptible plants showed differential expression of genes in response to fungal challenge. A putative disease resistance gene, jg24539.t1, was identified in the *L. scoparium* hypersensitive response phenotype family. Expression of this gene may

be associated with the phenotype and could be important for further understanding the plant hypersensitive response to *A. psidii* challenge. Differential expression of pathogen genes was found between samples taken 24 and 48 hpi, but there were no significant differences in pathogen gene expression that were associated with the three different plant leaf resistance phenotypes. There was a significant decrease in the abundance of fungal transcripts encoding three putative effectors and a putative carbohydrate-active enzyme between 24 and 48 hpi, suggesting that the encoded proteins are important during the initial phase of infection. These transcripts, or their translated proteins, may be potential targets to impede the early phases of fungal infection by this wide-host-range obligate biotrophic basidiomycete.

**Keywords:** disease resistance, diseases in natural plant populations, bioinformatics, fungal pathogens, genomics, pathogen effectors

*Austropuccinia psidii* (G. Winter) Beenken comb. nov. (Beenken 2017) is the causal agent of myrtle rust disease on a wide range of Myrtaceae around the world (480 species in 69 genera; Soewarto et al. 2019a). This obligate, biotrophic basidiomycete was first described by Winter (1884) as *Puccinia psidii* G. Winter on the leaves of *Psidium guajava* L. (= *Psidium pomiferum*) in Brazil and has a number of synonyms, including *Bullaria psidii*, *Dicaeoma psidii*, and *Uredo rangeli* (Carnegie and Giblin 2014). *A. psidii* is an invasive fungal pathogen that is now widely dispersed and found throughout the American continent and Caribbean, Hawaii, Asia, Australia, New Caledonia, Aotearoa (New Zealand), and South Africa (Carnegie and Pegg 2018). Stewart et al. (2018) identified nine distinct genetic clusters (C1 to C9) of *A. psidii* based on multilocus genotype analysis and host range. The pandemic strain, comprising the C1 and C3 clusters, is now causing the functional extinction of Myrtaceae from the subtropical east coast of Australia (Fensham et al. 2020) and presents a generational extinction threat

to a range of Australian east coast tropical species (Fensham and Radford-Smith 2021).

Myrtle rust, resulting from infection by the pandemic strain (du Plessis et al. 2019), was first found in Aotearoa on *Metrosideros kermadecensis* (Kahikā Rangitāhua, Kermadec pōhutukawa) growing on Rangitāhua (Raoul Island), the largest of the Kermadec Islands, in April 2017. A month later, the pathogen was found in Kerikeri in Te Ika-a-Māui (North Island) on *M. excelsa* (pōhutukawa; Ho et al. 2019). The pathogen is now widely established throughout most of Te Ika-a-Māui and has also been found on the west coast of Te Waipounamu (South Island) and as far south as Ōtautahi (Christchurch) on the east coast of Te Waipounamu (Anonymous 2021).

Currently, there are 18 recognized endemic and indigenous Myrtaceae in six genera in Aotearoa. These species are considered taonga (treasure) by Māori, who have a role in providing guardianship over these species. The reduction of the 27 species noted in Smith et al. (2020) is a consequence of the findings by Heenan et al. (2024) from a single-nucleotide polymorphism analysis of *Kunzea* that resulted in recognition of a single species, *K. ericoides*, in Aotearoa and the findings of a single-nucleotide polymorphism analysis of *Leptospermum scoparium* (mānuka) by Chagné et al. (2023), which provided “little support for taxonomic revision and subdividing *L. scoparium* into segregate species” within Aotearoa, negating the recent taxonomic determinations of *L. repo* (de Lange and Schmid 2021) and *L. hoipolloi* (Schmid et al. 2023). These 18 endemic and indigenous species are susceptible to infection by the pandemic strain of *A. psidii* (Smith et al. 2020). The pandemic strain of *A. psidii* has now caused the localized extinction of *Lophomyrtus bullata* (ramarama) in the East Cape region of Te Ika-a-Māui (Gifford 2021). Additionally, some New Zealand Myrtaceae are also susceptible to infection by the South

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African strain (Roux et al. 2016) of *A. psidii* (Soewarto et al. 2021). Resistance to this pathogen is present in some species, in particular mānuka (*L. scoparium*) and kānuka (formerly *Kunzea robusta* and *K. linearis*, now *K. ericoides*); however, the percentage of resistant plants in the provenances tested is relatively low (Smith et al. 2020). Additionally, both the leaf and stem of *L. scoparium* and *K. ericoides* are infected by the pathogen: Analysis of the phenotype distribution of leaf/stem infection in plants from a range of sibling families suggested that the leaf and stem resistances are the result of independent disease resistance mechanisms (Smith et al. 2020).

Obligate biotrophic pathogens specifically interact with, and manipulate, their hosts to obtain resources for reproduction without triggering host defence responses. Flor (1942) first described the genetics of these interactions in the *Melampsora lini*–*Linum usitatissimum* pathosystem leading to the gene-for-gene hypothesis (Flor 1955) that defined the genetics of the specificity of the pathogen–host interaction. The extensive host range of the pandemic strain of *A. psidii* presents a significant challenge to understand the basis of pathogenicity and the mechanisms that this obligate biotrophic fungus utilizes to infect and reproduce on its wide range of hosts (Soewarto et al. 2019a). Loci that are associated with resistance to *A. psidii* have been identified in different Myrtaceae. The first locus reported, *Puccinia psidii* resistance gene 1 (Ppr1) (Junghans et al. 2003b), has been successfully used in the Brazilian *Eucalyptus grandis* breeding program, although a new race of *A. psidii* has been reported to have now broken that resistance (Almeida et al. 2021). Four additional independent resistance loci were found in *Eucalyptus globulus*: Ppr2 and Ppr3 were associated with disease symptom expression, and Ppr4 and Ppr5 were associated with hypersensitive resistance (Butler et al. 2016). The presence of these resistance loci, although beneficial, is intriguing, as the pathogen does not share co-evolutionary history with most of its current hosts.

Results from a range of investigations to understand the molecular/biochemical basis of host plant resistance to *A. psidii* have been reported including metabolic analyses (Moffitt et al. 2022; Sekiya et al. 2021) and transcriptomic analyses of *Arillastrum gum-miferum*, *Syzygium longifolium*, and *Tristanopsis glauca* (Soewarto et al. 2019b), *E. grandis* (Santos et al. 2020; Swanepoel et al. 2021, 2023), *Melaleuca quinquenervia* (Hsieh et al. 2018), and *Syzygium luehmannii* (Tobias et al. 2018). Several putative host resistance mechanisms were identified in these studies, including overexpression of receptor-like kinases, nucleotide-binding site leucine-rich repeat proteins (Hsieh et al. 2018; Tobias et al. 2018), protein kinase leucine-rich receptors (Santos et al. 2020), endochitinases (Soewarto et al. 2019b), and brassinosteroid-mediated signaling genes (Swanepoel et al. 2021). These studies have largely focused on the plant response to infection. Two fungal transcripts, a homolog of a fungal cellulase (P07982) and a homolog of a “fungal plant-induced rust protein” (O00057), were noted in samples taken 5 days postinoculation (Hsieh et al. 2018). A recent study identified 890 differentially expressed *A. psidii* genes in an *E. grandis* infection model, but only one gene was significantly differentially expressed at 1 day postinoculation (Swanepoel et al. 2023). No genes were identified at 12 h postinoculation (hpi) or 2 days postinoculation. Additionally, in the above studies, only the Swanepoel et al. (2021, 2023) investigations used plants for which the myrtle rust disease resistance rating had been predetermined. In those studies, only immune resistant and highly susceptible plants were used, and hypersensitive resistant plants were not included.

Tobias et al. (2016) proposed that the most likely mechanism for the resistance levels found in Australian Myrtaceae was “a common Myrtaceae effector hub” that “on modification, triggers host recognition and response” as the “proportions of resistant plants are problematic to explain without a co-evolved selective pressure.” In this study, we sought evidence to test this hypothesis using Aotearoa providence *L. scoparium* to gain an initial understanding of the molecular basis of host resistance following challenge

by the pandemic strain of *A. psidii*. This study also sought to understand the basis of fungal pathogenicity on these *L. scoparium* plants for which the resistance phenotype was already known; in particular, did the pathogen gene expression change after inoculation onto immune resistant, hypersensitive resistant, and susceptible *L. scoparium* plants?

## Materials and Methods

### *L. scoparium* plants and experimental design

Plants were selected from four seed families sourced from plants from the East Cape region of Te Ika-a-Māui that had been previously assessed for leaf and stem resistance as described in Smith et al. (2020). These shade-house potted plants were cut back to remove infected material and regrown for 8 weeks, as the pathogen is not systemic, as per Swanepoel et al. (2021). All selected plants were stem infection resistant (S1). In total, 24 plants from four seed families (F01, F02, F03, and F04) were selected that had been leaf resistance phenotyped as per Smith et al. (2020) (L1, immune resistant; L2, hypersensitive resistant; L5, highly susceptible). Twelve were L1S1 (leaf immune resistant, stem resistant) plants, four L2S1 (leaf hypersensitive resistant, stem resistant) plants, and eight L5S1 (leaf highly susceptible, stem resistant) susceptible plants (Table 1). Half of the plants were randomly assigned as controls, and the other half were assigned to the inoculated group, with plants from each of the four families in both the inoculated and control treatments.

### Inoculation

After regrowth, the control plants were sprayed with the inoculation solution (two drops of Tween® 20 per 100 ml of sterile distilled water). The inoculated plants were sprayed with inoculation solution containing  $1 \times 10^5$  *A. psidii* urediniospores per milliliter. The inoculated seedlings were then covered with plastic sheeting, and hot tap water (60°C) was applied to the lower plastic sheet, creating a sealed environment to maintain humidity and leaf wetness. The covered plants were then placed into a controlled-environment chamber in darkness at 18°C and 80% relative humidity. After 24 h, the plastic coverings were removed, and seedlings were transferred into a shade house and watered for 10 min twice daily, as described by Smith et al. (2020).

### RNA preparation and sequencing

Each plant was sampled 24 and 48 hpi by removing 8 to 12 leaves from directly below the stem apical meristem, which were immediately snap frozen in liquid nitrogen and then ground in liquid nitrogen with a mortar and pestle prior to total RNA extraction using the Norgen Plant/Fungi Total RNA Purification Kit, including the optional chloroform extraction step. The RNA preparation quality was analyzed by the Australian Genome Research Facility (AGRF, <https://www.agrf.org.au/>) Melbourne prior to single-lane Illumina NovaSeq 6000 S4 sequencing with 150-bp paired-end reads. Image analysis was performed in real time using the NovaSeq Control Software v1.6.0 and Real Time Analysis v3.4.4, running on the instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data for 150-bp paired end reads (Illumina, San Diego, CA).

### Sequencing data quality checking, clean-up, and mapping to the reference genome

Ribosomal RNA sequences were removed from the raw RNA sequencing data using SortMeRNA (version 2.1b; Kopylova et al. 2012). Adaptors, low-quality sequences, and homo-polymers were removed using Trimmomatic (version 0.36; Bolger et al. 2014). FastQC (version 0.11.7; Andrews 2010) and MultiQC (version 1.7; Ewels et al. 2016) were used throughout the data processing steps to ascertain the integrity of the data. Picard tools (version 2.9.4; Broad Institute, <https://broadinstitute.github.io/picard/>) was used to add metadata to the read sets, and the STAR aligner (version 2.6.1d;

Dobin et al. 2013) was used to align sequence reads to the *L. scoparium* genome (Thrimawithana et al. 2019; permission obtained 29 March 2023 from Kaitiaki Māori via the Aotearoa Genomic Data Repository, <https://data.agdr.org.nz/>) and to the *A. psidii* genome (Tobias et al. 2021). Count data for the expressed genes were obtained using HTSeq count (version 0.9.1; Anders et al. 2015).

### Differential gene expression analysis

DESeq2 (Love et al. 2014) was used for the differential expression analysis. R (version 4.0) was used for the analysis, and the results were visualized using ggplot2 (version 3.3.5; Wickham 2016). Treatment and plant family were combined into one factor, referred to as a combo. The DESeq2 design for the *L. scoparium* data was as follows: design = ~time + combo + time:combo. This formula modeled the difference between the two time points, the difference between the treatments and plant families (represented by the combo factor), and any treatment-plant family differences over time. The *A. psidii* design was simpler, and only the difference at the two time points was modeled (design = ~time). The false discovery rate/alpha was set to 0.1. All reported *P* values are the adjusted *P* values as generated by the DESeq2 analysis. Principal component analysis (PCA) was performed on the 100 genes with the smallest adjusted *P* value (i.e., with the most significant changes in gene expression) after variance stabilizing transformation, and hierarchical clustering was performed on the data from all genes following variance stabilizing transformation.

### BLAST

The predicted *L. scoparium* (Thrimawithana et al. 2019) and *A. psidii* (Tobias et al. 2021) genes were compared against sequences in the SwissProt database ("UniProt: a worldwide hub of protein knowledge," 2018) using the BLAST algorithm through the BLAST+ command line application (Camacho et al. 2009) to identify sequences with putative functions that share sequence similarity to the genes.

### Pathway enrichment analysis

The predicted protein sequences from the *L. scoparium* and *A. psidii* genomes were submitted to the Kyoto Encyclopedia of Genes and Genomes annotation server to obtain Kyoto Encyclopedia of Genes and Genomes ontologies (Kanehisa 2019; Kanehisa and Goto 2000; Kanehisa et al. 2019). The pathway analysis was done using gage (version 2.37.0; Luo et al. 2009) and the results visualized using pathview (version 1.28.0; Luo and Brouwer 2013).

### Co-expression analysis

Co-expression analysis was undertaken by creating correlation matrices, using DESeq2 results and the R package qgraph (Epskamp et al. 2012). Data were visualized using correlation graphs for selected gene groups of interest.

### Carbohydrate-active enzymes (CAZymes) identification

dbCAN3 (Zhang et al. 2018) was used to identify CAZymes in the *A. psidii* genome. Additional CAZymes were identified through manual analysis of gene sequences in the genome.

## Results

### Sequencing

**RNA sequencing libraries.** In total, 48 leaf samples were taken over the two time points and RNA extracted. One F02\_L1S1 sample RNA extraction failed, and two RNA library creations failed (F02\_L1S1 and F04\_L5S1), resulting in 45 sequence libraries over the four families and two time points (Table 1). Three hundred and fifty-three gigabytes of data containing 1.167 billion reads was created (the average number of reads per sample was 25.90 million [range 22.84 to 31.24] and the average data yield per sample was 7.83 GB [range 6.90 to 9.43; Supplementary Table S1]).

### *L. scoparium* differential gene expression

**Changes in gene expression are influenced by *L. scoparium* family, time, and treatment.** PCA and hierarchical clustering methods were used to understand the underlying substructure of the *L. scoparium* gene expression data. These analyses suggested that the differential gene expression patterns were strongly linked to sampling time, plant family, and treatment. These factors were subsequently included in the final DESeq2 design. The PCA plot from the final DESeq2 analysis design is shown in Figure 1A to D. The sample points are colored by family, sampling time (hpi), treatment, and phenotype. The first principal component accounts for 26% of the variation and separates the samples by family, and the second principal component accounts for 12% of the variation, and sampling time and treatment appear to drive separation along this component. Phenotype does not provide clear separation, with a mixed cluster of L1S1 and L2S2 samples in the center of the plot.

**Differences between uninoculated *L. scoparium* families.** There are differences in gene expression between the different *L. scoparium* families, even without *A. psidii* inoculation (control group). The number of significantly differentially expressed genes for each family for different comparisons are in Table 2. The up- and downregulated genes for each family compared with F04\_L5S1 are listed in Supplementary File S1. Nine genes were identified as differentially expressed in more than one family; a Venn diagram illustrating this is shown in Figure 1E, and the nine genes differentially expressed by F01\_L1/2S1, F02\_L1S1, and F03\_L2S1 are listed in Table 3. No metabolic pathways were identified as having significant changes in gene expression using gage pathway analysis.

***L. scoparium* families respond to inoculation with *A. psidii*.** The four *L. scoparium* families had different responses at both 24 and 48 hpi with *A. psidii*. The up- and downregulated genes for

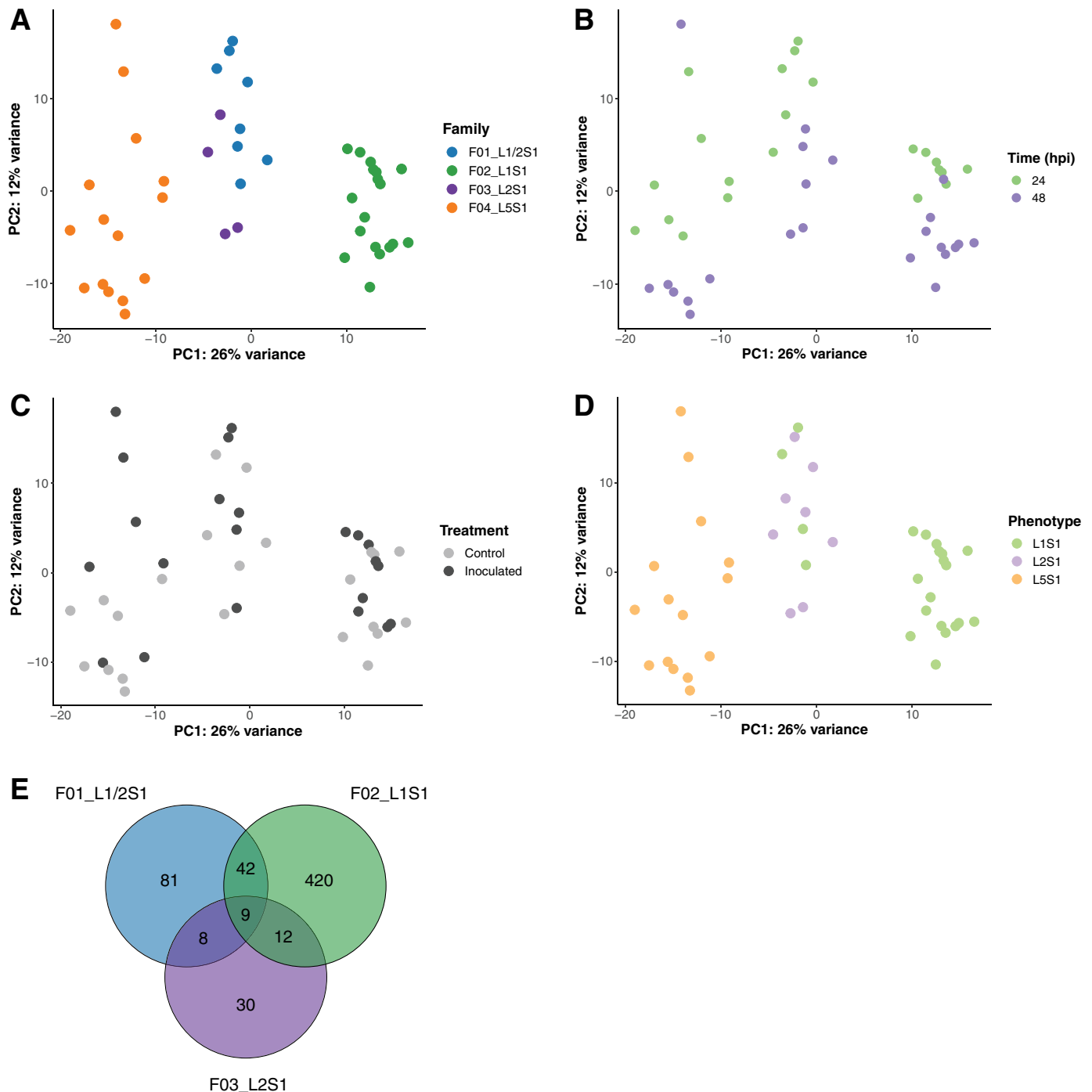
TABLE 1. The assignment of *Leptospermum scoparium* seed family plants to treatments and successful RNA sequencing (RNAseq) libraries based on predetermined leaf infection resistance phenotype<sup>a</sup>

Phenotype	Treatment	Family	Number of plants	Number of RNAseq libraries (24 hpi)	Number of RNAseq libraries (48 hpi)
Immune resistant (L1S1)	Inoculated	F01_L1/L2S1	1	1	1
		F02_L1S1	5	4	5
	Control	F01_L1/L2S1	1	1	1
		F02_L1S1	5	4	5
Hypersensitive resistant (L2S1)	Inoculated	F03_L2S1	1	1	1
		F01_L1/L2S1	1	1	1
	Control	F03_L2S1	1	1	1
		F01_L1/L2S1	1	1	1
Susceptible (L5S1)	Inoculated	F04_L5S1	4	4	3
	Control	F04_L5S1	4	4	4
Total			24	22	23

<sup>a</sup> Seed families: F01, F02, F03, and F04. Leaf resistance phenotype L1, immune resistant; L2, hypersensitive resistant; and L5, highly susceptible. Stem resistance phenotype S1, resistant. hpi, hours postinoculation.

each family comparing the inoculated plants with the control plants at 24 hpi are listed in Supplementary File S2. The up- and down-regulated genes for each family comparing the inoculated plants with the control plants at 48 hpi are listed in Supplementary File S3. Only a small number of genes (3 to 14) were differentially expressed at 24 hpi for all families, whereas 1,343 genes were up-regulated and 373 downregulated by F04\_L5S1 (leaf susceptible,

stem resistant) at 48 hpi (Table 2). F01\_L1/2S1 (leaf immune and hypersensitive response resistant, stem resistant) had a single up-regulated gene, F02\_L1S1 (leaf immune resistant, stem resistant) had nine upregulated and four downregulated, and no differentially expressed genes were identified for F03\_L2S1 (leaf hypersensitive response resistant, stem resistant) at 48 hpi. The one upregulated gene (jg20441.t1) from F01\_L1/2S1 was also upregulated in both



**Fig. 1.** Differentially expressed *Leptospermum scoparium* genes are linked to family, sampling time, and treatment. Principal component analysis (PCA) plots were used to understand *L. scoparium* sample similarity and underlying substructures in the data. The PCA plot was generated using variance-stabilizing transformation of gene expression count data from the 100 genes with the most significant changes in gene expression when comparing samples from 48 and 24 h postinoculation (hpi). The sample points are colored based on **A**, family; **B**, sampling time (hpi); **C**, treatment (inoculated or control); and **D**, phenotype (L1, immune leaf resistance; L2, hypersensitive leaf resistance; L5, leaf susceptible; and S1, stem resistance). The samples separate based on family along the first PC axis and sampling time along the second PC axis. Treatment also appears to contribute to the variance in the second PC. The families are F01, F02, F03, and F04. The phenotypes are L1S1, leaf immune resistance, stem resistant; L2S1, leaf hypersensitive resistant, stem resistant; and L5S1, leaf highly susceptible, stem resistant. Family F01 contains both L1S1 and L2S1 plants. **E**, Venn diagram showing the number of common differentially expressed genes across families with resistance to *Austropuccinia psidii* when compared with the susceptible family (F04\_L5S1). Each family has a unique gene expression profile. Nine transcripts are shared by all families. These differences in gene expression were observed in the control plants that were not inoculated with *A. psidii*.

the F02\_L1S1 and F04\_L5S1 families. The protein sequence from this gene has a top blastp match to a BTB/POZ domain-containing protein (AT2G30600) from *Arabidopsis thaliana*. There was no other overlap in differentially expressed genes at either the 24 or 48 hpi timepoints. The gene jg24539.t1 was significantly upregulated in F03\_L2S1 plants inoculated with *A. psidii* compared with control plants at 24 hpi (Supplementary File S2). The blastp result suggests this gene contains a leucine-rich repeat and has similarity to disease resistance protein RUN1-like isoform X1 (XP\_018717429.1) and X2 (XP\_018717437.1) from *E. grandis* and other disease resistance proteins found in a range of *Syzygium* species.

### A. *psidii* differential gene expression

**Gene expression changes over time.** Hierarchical clustering and PCA were used to assess the similarities between samples and to identify determinants that differentiated them. Both the heat map and PCA plot in Figure 2A and B show sampling time to be a major differentiating factor. The 24 hpi samples are highly similar and tightly clustered, whereas the 48 hpi samples are less tightly clustered but are separate from the 24 hpi samples. Both putative CAZymes and putative effectors were present in the differential gene expression analysis of the top 10 fungal genes ranked by adjusted *P* value after analysis of differential gene expression between the two sampling time points (24 and 48 hpi) in Figure 2C.

**Host plant phenotype or family has a limited impact on fungal gene expression.** The *A. psidii* gene expression data were analyzed by comparing the 24 with the 48 hpi sampling point. The expression profiles of both putative effectors (Fig. 2D) and putative CAZymes (Fig. 2E) changed between 24 and 48 hpi. Both the effector and the CAZyme heatmaps appear to independent of both plant family and resistance phenotype, suggesting that the changes in the pathogen expression profiles in the first 48 h are not in response to any external criteria (e.g., a plant hypersensitive response). When the host plant family was included as a factor in the analysis, only 134 *A. psidii* genes were identified as significantly differentially expressed after inoculation to plants in the F02\_L1S1 and F04\_L5S1 families (Table 4). No significant differentially expressed fungal genes were identified after inoculation to plants in the F03\_L2S1 and F04\_L5S1 families. When sampling time was the only factor used in the analysis, 151 upregulated and 332 downregulated fungal genes were identified. The DESeq2 design using only sampling time as a factor was subsequently chosen as the best representation of the data and is used from here on.

**Changes in *A. psidii* gene expression at 24 compared with 48 hpi.** Transcripts for 16 predicted effectors were more abundant at 24 compared with 48 hpi (Fig. 2D). Twelve predicted CAZymes were more abundant at 24 compared with 48 hpi. Additionally, there were three predicted CAZymes with the opposite

expression pattern (APSI\_P010.11473.t1, APSI\_P021.13488.t1, and APSI\_P013.4183.t1; Fig. 2E). The 10 genes with the greatest upregulation and the 10 with the greatest downregulation at 48 compared with 24 hpi are listed in Table 5. The full gene list is in Supplementary File S4. Investigation of gene pathways using gage (Luo et al. 2009) identified several upregulated pathways relating to increased fungal growth at 48 compared with 24 hpi. This included the ribosome, biosynthesis of secondary metabolites, biosynthesis of amino acids, and oxidative phosphorylation pathways. Only the ribosome pathway was significantly upregulated (*P* value  $1.48 \times 10^{-21}$ ). No significantly downregulated pathways were identified. A correlation analysis to identify patterns in gene analysis was performed on the 10 genes with the smallest adjusted *P* value (Fig. 2C). APSI\_P010.11473.t1, a putative patulin synthase and a CAZyme, expression was negatively correlated with two other CAZymes and three effectors. The three effectors' expression levels were all positively correlated with each other.

## Discussion

### L. *scoparium* response to infection

This is the first report of the differential transcriptomic response of *L. scoparium* (mānuka) plants to challenge by germinating urediniospores of *A. psidii*. Similar to previous studies (Swanepoel et al. 2021, 2023), the resistance phenotypes of the plants in this study were previously characterized. In contrast to those studies, where only immune resistant and highly susceptible plants were selected for transcriptomic analysis (ratings 1 and 5, respectively, on the modified Junghans scale; Junghans et al. 2003a), hypersensi-

TABLE 3. *Leptospermum scoparium* genes that are differentially expressed by F01\_L1/2S1, F02\_L1S1, and F03\_L2S1 compared with F04\_L5S1 at 24 h postinoculation in uninoculated plants<sup>a</sup>

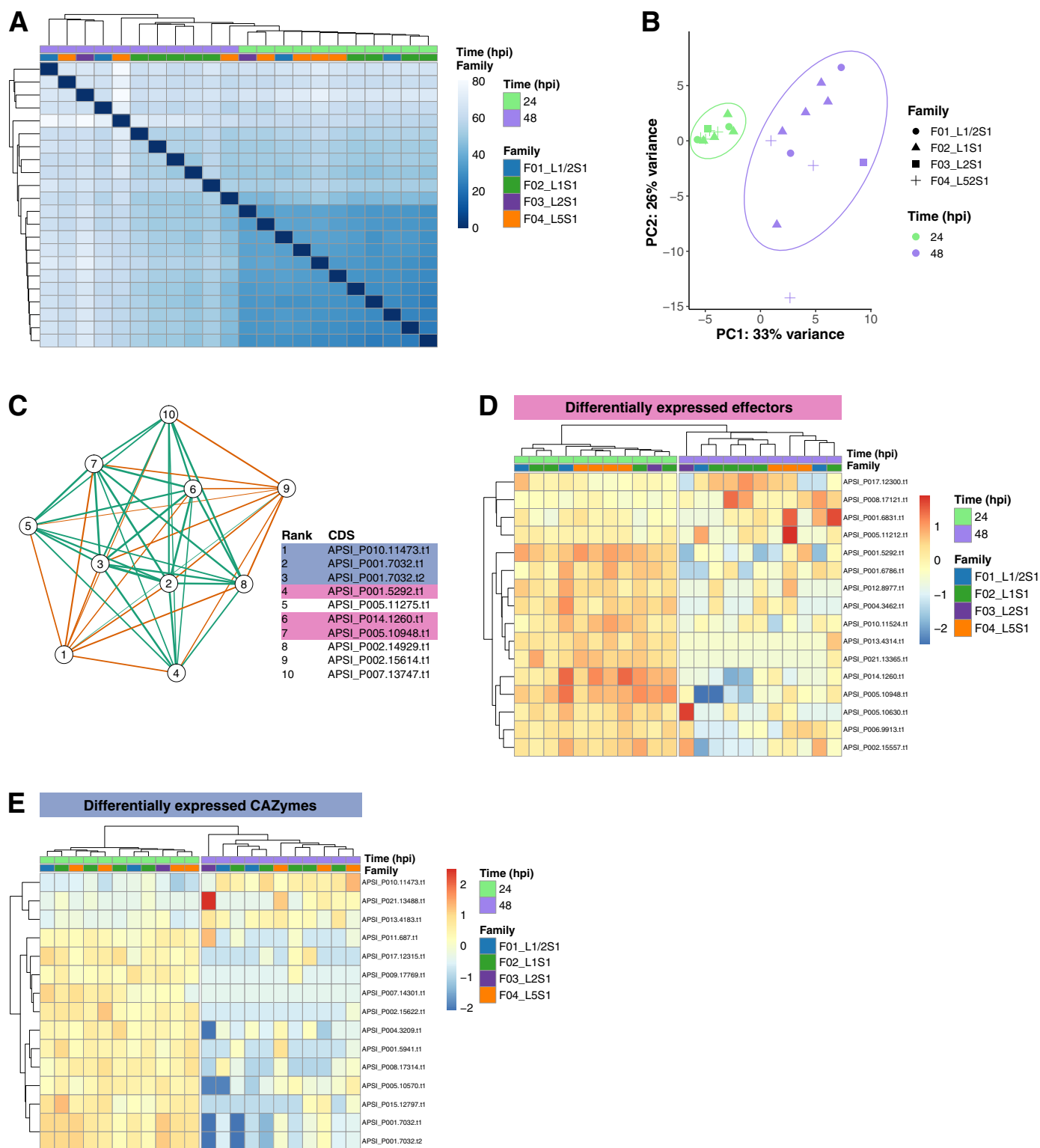
Coding sequence	Top blastp match from SwissProt database	E-value
jg27885.t1	SNF2 domain-containing protein CLASSY 2	0.0
jg6587.t1	Potassium transporter 10	0.29
jg7658.t1	Aspartic proteinase CDR1	$2.17 \times 10^{-102}$
jg8213.t1	Alginate lyase	$9.95 \times 10^{-178}$
jg11269.t1	Putative clathrin assembly protein At5g35200	0.0
jg3844.t1	Coiled-coil domain-containing protein 124	$4.28 \times 10^{-19}$
jg23001.t1	Probable pectinesterase 8	$4.48 \times 10^{-165}$
jg5220.t1	Probable protein phosphatase 2C 4	1.9
jg15853.t1	E3 ubiquitin-protein ligase RFI2	$2.01 \times 10^{-61}$

<sup>a</sup> Seed families: F01, F02, F03, and F04. Leaf resistance phenotypes: L1, immune resistant; L2, hypersensitive resistant; and L5, highly susceptible. Stem resistant phenotype S1, resistant. The F01 family contained both L1 and L2 plants, whereas the F04 family only contained L5 plants. A putative gene function is listed based on the top blastp match using the SwissProt database.

TABLE 2. Number of significantly up- and downregulated genes in *Leptospermum scoparium*<sup>a</sup>

Comparison	Supplementary file	Family	Number of upregulated genes	Number of downregulated genes
Uninoculated plants compared with F04_L5S1 (susceptible) at 24 h	S1	F01_L1/2S1	71	69
		F02_L1S1	222	261
		F03_L2S1	23	36
Inoculated versus uninoculated plants at 24 h	S2	F01_L1/2S1	6	4
		F02_L1S1	3	5
		F03_L2S1	14	6
		F04_L5S1	6	6
Inoculated versus uninoculated plants at 48 h	S3	F01_L1/2S1	1	0
		F02_L1S1	9	4
		F03_L2S1	0	0
		F04_L5S1	1,343	372

<sup>a</sup> Results for several comparisons were extracted from the DESeq2 analysis. Significant genes were defined as genes with a  $\geq 2$ -fold change in gene expression with an adjusted *P* value  $< 0.1$ . Seed families: F01, F02, F03, and F04. Leaf resistance phenotype L1, immune resistant; L2, hypersensitive resistant; L5, highly susceptible. Stem resistant phenotype S1, resistant. The F01 family contained both L1 and L2 plants, whereas the F04 family contained only L5 plants.



**Fig. 2.** Identification of differentially expressed *Austropuccinia psidii* genes. **A**, Hierarchical clustering and heatmap of *A. psidii* gene expression data. Analysis of *A. psidii* sample similarity and underlying substructures in the data was performed using variance-stabilizing transformation of gene expression count data. The color gradient corresponds to the correlation of gene expression for sample pairs. The samples cluster together based on the sampling time. **B**, Principal component analysis (PCA) plot generated using variance-stabilizing transformation of gene expression count data from the 100 *A. psidii* genes with the most significant changes in gene expression when comparing samples from 48 and 24 h postinoculation (hpi). The sample points are colored based on sampling time (hpi), and the shapes depict the different families. The samples cluster based on sampling time. **C**, Correlation network diagram of the top 10 *A. psidii* genes ranked by adjusted *P* value following differential gene expression analysis comparing 24 and 48 hpi. No cutoff was applied to the fold change. Green edges represent positive correlations, orange edges represent negative correlations, and the edge width represents the strength of the correlation. Genes highlighted blue in the legend are carbohydrate-active enzymes (CAZymes), and genes highlighted pink are effectors (as identified in Tobias et al. 2021). The expression profiles of **D**, effectors and **E**, CAZymes change over time. The variance-stabilized count data for effector and CAZyme genes with significant changes in gene expression at 48 compared with 24 hpi was used in a hierarchical cluster analysis that is depicted in the heatmaps. The family (F01, F02, F03, and F04) phenotypes are L1S1, leaf immune resistant, stem resistant; L2S1, leaf hypersensitive resistant, stem resistant; and L5S1, leaf highly susceptible, stem resistant.

tive resistant plants (rating 2 on the modified Junghans scale; Smith et al. 2020) were also included in this study. Santos et al. (2020) used clones of two well-studied plant *E. grandis* genotypes (resistant CLR385 and susceptible CLR220), whereas in other myrtle rust gene expression studies, the resistance phenotype was determined during the study itself (Hsieh et al. 2018; Tobias et al. 2018) or observed at sample collection (Soewarto et al. 2019b). Additionally, in this study and that of Santos et al. (2020), the genetic relationship of the plants (seed family siblings or clones) was known (Table 1). For this pathogen-host study, knowing the resistance phenotype of the plants was important, as *L. scoparium* has two tissue-specific resistances, leaf and stem, that are hypothesized to be genetically independent based on disease resistance phenotype distributions within, and between, seed families (Smith et al. 2020). To minimize the complexity of the experimental design and subsequent transcript analyses, only stem infection resistant (S1) *L. scoparium* plants were selected for this study. Stem infection resistance is hypothesized to be an immune resistance, as no equivalent to L2 (leaf hypersensitive resistance) was observed during the original phenotyping (Table 2 in Smith et al. 2020).

Six Myrtaceae have been assessed in previous myrtle rust transcriptomic studies: *E. grandis*, *A. gummiferum*, *S. longifolium*, *T. glauca*, *M. quinquenervia*, and *S. luehmannii* (Hsieh et al. 2018; Santos et al. 2020; Soewarto et al. 2019b; Swanepoel et al. 2021, 2023; Tobias et al. 2018). This study is the first to use *L. scoparium* and the first myrtle rust transcript study to investigate the response to fungal challenge by sibling plants from known seed families.

TABLE 4. Number of significantly up- and downregulated genes in *Austropuccinia psidii*<sup>a</sup>

Analysis (48 compared with 24 hpi)	Samples	Number of upregulated genes	Number of downregulated genes
With family and time as factors	F01_L1/2S1	0	0
	F02_L1S1	21	38
	F03_L2S1	0	0
	L04_L5S1	53	22
With time as a factor	All samples	151	332

<sup>a</sup> One analysis included host plant family and time as factors, whereas a second analysis only included time as a factor. Significant genes were defined as genes with a  $\geq 2$ -fold change in gene expression at 48 h postinoculation (hpi) compared with 24 hpi with an adjusted *P* value  $< 0.1$ .

Although the *L. scoparium* siblings are not biological replicates, as previously noted by Smith et al. (2020), the original seed collected from the mother plants is considered to have been open pollinated, as Myrtaceae have late-acting self-incompatibility (Gibbs 2014), although self-compatibility exceptions have been noted in some Myrtaceae (Schmidt-Adam et al. 1999). Thus, the *L. scoparium* plants grown from seed from a seed family and used in this study are believed to have a common maternal genetic base with additional genetics via open pollination from one or more paternal plants.

Analysis of the top 100 *L. scoparium* significantly expressed genes confirmed that plant family was the factor with the strongest influence on the data structure (Fig. 1). Differences between the families were wide ranging, with only nine genes common between the resistant families (F01\_L1/2S1, F02\_L1S1, and F03\_L2S1) and the susceptible family (F04\_L5S1; Fig. 1E; Table 3). This finding is consistent with the results found using single-nucleotide polymorphism-based methods (Chagné et al. 2023; Koot et al. 2022), where geographic populations were evident in the genomic data from *L. scoparium* plants sampled across New Zealand.

The low number of differentially expressed genes identified for F01\_L1/2S1 and F03\_L2S1 are likely due to the small number of samples in each group. However, it is interesting that these families cluster together in the PCA plot (Fig. 1A to D) despite the mix of phenotypes. It is possible that the F01\_L1/2S1 plants with an L1S1 phenotype also carry L2S1 immune resistance genes, as do their L2S1 siblings and members of the F03\_L2S1 family. This L2 hypersensitive response would not be necessary or measurable if the plant also has genes for the L1 immune resistant phenotype. One gene (jg20441.t1) was upregulated in the F01\_L1/2S1, F02\_L1S1, and F04\_L5S1 families at 24 hpi when comparing inoculated with control plants. The predicted protein from this gene contains a BTB/POZ domain. These domains are key factors enabling protein–protein interactions, and proteins with BTB/POZ domains have a wide variety of roles in cells, including transcription repression, protein ubiquitination and degradation, and cytoskeleton regulation (Stogios et al. 2005). Further study of this gene and its protein will be required to understand if it has an important role in the response of *L. scoparium* to *A. psidii* infection. When inoculated with *A. psidii*, only a few genes showed a change in expression level in the immune resistant F02\_L1S1 plants (Table 2). This suggests that the basis of this resistance is preformed and is not reliant on plant recognition of infection and response. This limited change

TABLE 5. The 10 *Austropuccinia psidii* genes with the greatest up- and downregulation in expression at 48 h postinoculation (hpi) compared with 24 hpi<sup>a</sup>

Coding sequence	Top blastp match from SwissProt database	Log <sub>2</sub> fold change	Adjusted <i>P</i> value
Upregulated			
APSI_P020.4955.t1	4-Amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase	7.01	1.7E-05
APSI_P005.11227.t1	Trigger factor	6.46	1.8E-04
APSI_P004.3809.t1	Sodium/glucose cotransporter 4	4.91	1.2E-02
APSI_P021.13488.t1	Levanase	4.82	7.5E-04
APSI_P003.2293.t1	Endoribonuclease dcr-1	4.73	2.3E-03
APSI_P007.14480.t1	Protein adenyltransferase SelO	4.66	3.3E-02
APSI_P007.14353.t1	Glutamate racemase	4.39	4.1E-02
APSI_P017.12307.t1	40S ribosomal protein S0	4.17	1.6E-05
APSI_P012.8820.t1	Calcium-transporting ATPase 1, plasma membrane-type	4.16	1.1E-05
APSI_P020.4940.t1	RNA-binding posttranscriptional regulator cip2	4.08	5.3E-03
Downregulated			
APSI_P007.14121.t1	Zinc finger CCCH domain-containing protein 4	−3.70	1.5E-05
APSI_P009.17743.t1	UPF0307 protein AHA_3937	−3.56	9.3E-05
APSI_P002.15622.t1	Mannosyl-oligosaccharide alpha-1,2-mannosidase	−3.44	8.9E-05
APSI_P013.4407.t1	Proline–tRNA ligase	−3.12	1.3E-06
APSI_P002.15849.t1	Probable serine/threonine-protein kinase PBL19	−3.09	5.1E-04
APSI_P004.2910.t1	Putative pre-16S rRNA nuclease	−3.08	4.8E-04
APSI_P021.13365.t1	Ribonuclease PH <sup>b</sup>	−3.05	1.0E-03
APSI_P002.15017.t1	Chorion transcription factor Cf2	−3.05	1.6E-05
APSI_P015.13082.t1	Protein-L-isoaspartate O-methyltransferase	−2.97	5.7E-05
APSI_P007.14301.t1	Pectinesterase	−2.90	1.8E-02

<sup>a</sup> A putative gene function is listed based on the top blastp match using the SwissProt database.

<sup>b</sup> Identified as an effector by Tobias et al. (2021).

in gene expression in leaf immune resistant plants was similar to that observed in *E. grandis* by Santos et al. (2020). The susceptible F04\_L5S1 plants had numerous differentially expressed genes, suggesting the plant had sensed the presence of the pathogen, but ultimately, the response was not successful. This contrasts with the findings of Tobias et al. (2018) and Swanepoel et al. (2021), where a greater number of differentially expressed genes were observed in the resistant plants of *S. luehmannii* and *E. grandis* compared with the susceptible plants. The different responses between these experiments could be due to the different plant species or to the limited sampling points within each experiment. Further research investigating gene expression changes over more time points may reveal plants with different disease phenotypes responding differentially to infection in this non-coevolved pathosystem.

This study included plants with a hypersensitive resistance phenotype (rating 2 on the modified Junghans scale; Smith et al. 2020). Although some differentially expressed genes were found at 24 hpi when inoculated plants were compared with control plants, no differentially expressed genes were identified at 48 hpi. Despite the small sample size in these experiments, we were able to identify one potential disease resistance gene that may be associated with the hypersensitive response in these plants. The gene, jg24539.t1, was identified as a potential disease resistance gene linked to the L2 hypersensitive response phenotype of the F03\_L2S1 family. Similar genes have been annotated in the genomes of *E. grandis* and other *Syzygium* species. These genes have similarity to the grape “resistance to *Uncinula necator*” (*MrRUN1*) gene, which confers resistance to the fungal pathogen *U. necator* (synonym *Erysiphe necator*), the causal agent of powdery mildew (Feechan et al. 2013). Future studies using more plants with this phenotype could enable the full hypersensitive resistance mechanism to be elucidated. However, the low abundance of hypersensitive plants in *L. scoparium* populations will make elucidation challenging, as only 2.3% of the *L. scoparium* families screened by Smith et al. (2020) contained plants with the L2S1 resistance phenotype.

No transcripts associated with other forms of disease resistance were found in these analyses. For example, other studies had found transcripts associated with glutathione S-transferases (Hsieh et al. 2018) and changes to brassinosteroid signaling (Swanepoel et al. 2021). Tobias et al. (2016) suggested that the range of responses by the different plant species challenged by *A. psidii* may reflect the lack of co-evolution between the plant hosts and this pathogen and that the most likely mechanism for the resistance levels found in Australian Myrtaceae was “a common Myrtaceae effector hub” that “on modification, triggers host recognition and response” as the “proportions of resistant plants are problematic to explain without a co-evolved selective pressure.” The results from this study that each resistant *L. scoparium* family (F01, F02, F03) has a unique gene expression profile, with only nine transcripts shared by all families (Fig. 1E), provides little support for the hypothesis of “a common Myrtaceae effector hub” as there is significant diversity of genetic responses in this single species to fungal challenge. A much larger study with many more families and species would be required to validate or refute this hypothesis.

### A. *psidii* gene expression

As noted previously, most of the previous *A. psidii*-host studies either did not note or reported only a limited number of fungal transcripts in their analysis. The *E. grandis* study by Swanepoel et al. (2023) reported 890 *A. psidii* transcripts, including 43 candidate effector protein genes at the 0.5-, 1-, 2-, and 5-day sample time points, with most reads mapping to the susceptible host samples taken 5 days after inoculation. The top 10 differentially expressed genes between 24 and 48 hpi in this study were not present in the top 100 most highly expressed *A. psidii* genes (based on fragments per kilobase of transcript per million reads mapped) in the susceptible or resistant host infections in the *E. grandis* infection study of Swanepoel et al. (2023). These two studies used different plant

species but did have two aligned sampling time points (24 h/1 day and 48 h/2 days). The different results from this study and that of Swanepoel et al. (2023) reflect one of the challenges of investigating this multi-host pathosystem. The top three differentially expressed fungal transcripts in this study encoded putative CAZymes, suggesting a key role for these proteins in enabling the pathogen to breach the plant cell wall. APSI\_P001.7032 has two predicted transcripts (t1 and t2): Both are predicted to be differentially expressed, but further work is required to confirm this result. Blastp identified a cellulase domain in APSI\_P001.7032 (pfam00150; glycosyl hydrolase family 5). Proteins with similarity to APSI\_P001.7032 are conserved across a range of fungi, including *Puccinia* spp., other Basidiomycota, and several Ascomycota species. The protein encoded by APSI\_P010.11473 is also conserved across a wide range of fungi. The blastp search against the SwissProt database had a match to patulin synthase, whereas a general blastp search detected a Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain and a GMC oxidoreductase domain (pfam00732/pfam05199). This gene family is predicted to be involved in the degradation of lignocellulose (Sützl et al. 2018). These putative functional domains in these CAZymes suggests they may have key roles in the initial phases of infection of the plant host.

Over 1,200 putative effector genomic sequences have been identified in the *A. psidii* genome and mapped to the two haploid genomes: 617 to haplotype 1 and 616 to haplotype 2 (Edwards et al. 2022). Effectors are relatively small proteins “that facilitate pathogen entry into the host interior, suppress plant immune perception, and alter host physiology for pathogen benefit” (Toruño et al. 2016). The presence of three predicted *A. psidii* effectors in the top 10 differentially expressed pathogen genes across all the inoculated plants samples suggests a key role for these proteins in successfully establishing infection. Interestingly, the top 10 differentially expressed genes found in this study are not present in the fragments per kilobase of transcript per million reads mapped ranked in Supplementary Table S2 (the top 100 most highly expressed *A. psidii* genes in the resistant interaction) nor in Supplementary Table S3 (the top 100 most highly expressed *A. psidii* genes in the susceptible interaction) in the findings of Swanepoel et al. (2023; [https://apsjournals.apsnet.org/doi/suppl/10.1094/PHYTO-07-22-0257-R/suppl\\_file/PHYTO-07-22-0257-R.sm2.xlsx](https://apsjournals.apsnet.org/doi/suppl/10.1094/PHYTO-07-22-0257-R/suppl_file/PHYTO-07-22-0257-R.sm2.xlsx)). BLAST searches of the three effector sequences did not identify any conserved domains; therefore, classic biochemical and interaction studies will be required to understand their role in the infection process. Genes with sequence similarity to APSI\_P001.5292.t1 and APSI\_P014.1260.t1 are present in other *Puccinia* species, suggesting a conserved function. APSI\_P005.10948.t1 appears to be unique to *A. psidii*.

These early expressed fungal genes found in this study are potentially critical to facilitate pathogen entry into the plant and manipulation of the plant cells for access to nutrition and for suppression of host detection and response systems. Further research to investigate this initial infection time period (up to 24 hpi) in detail is required to understand the function of these early expressed pathogen genes so that novel management techniques that directly target this pathogen can be developed.

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