Transcriptome Profiling of *Melaleuca quinquenervia* Challenged by Myrtle Rust Reveals Differences in Defense Responses Among Resistant Individuals

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ABSTRACT

Plants have developed complex defense mechanisms to protect themselves against pathogens. A wide-host-range fungus, *Austropuccinia psidii*, which has caused severe damage to ecosystems and plantations worldwide, is a major threat to Australian ecosystems dominated by members of the family Myrtaceae. In particular, the east coast wetland foundation tree species *Melaleuca quinquenervia*, appears to be variably susceptible to this pathogen. Understanding the molecular basis of host resistance would enable better management of this rust disease. We identified resistant and susceptible individuals of *M. quinquenervia* and explored their differential gene expression in order to discover the molecular basis of resistance against *A. psidii*. Rust screening of germplasm showed a varying degree of response, with fully resistant to highly susceptible

individuals. We used transcriptome profiling in samples collected before and at 5 days postinoculation (dpi). Differential gene expression analysis showed that numerous defense-related genes were induced in susceptible plants at 5 dpi. Mapping reads against the *A. psidii* genome showed that only susceptible plants contained fungal-derived transcripts. Resistant plants exhibited an overexpression of candidate *A. psidii* resistance-related genes such as receptor-like kinases, nucleotide-binding site leucine-rich repeat proteins, glutathione S-transferases, WRKY transcriptional regulators, and pathogenesis-related proteins. We identified large differences in the expression of defense-related genes among resistant individuals.

Additional keywords: plant defense, RNA-Seq.

Plants have developed complex and multilayered defense mechanisms to protect themselves against pathogens. The first layer of defense includes physical barriers such as cuticles, trichomes, and cell walls, as well as constitutively expressed secondary metabolites with antifungal and antibacterial properties (Naidoo et al. 2014). Preformed physical and chemical barriers are generally sufficient to shield the plant from most pathogens, though compatible pathogens may breach this line of defense by degrading the plant cell wall using hydrolytic enzymes, or directly penetrating the cell wall by feeding apparatus, as in the case of haustoria formation in biotrophic rust fungi (Underwood 2012). Hence, recognizing intruding pathogens is essential in mounting an effective defense.

Pathogen recognition in plants incorporates two styles of recognition factors, pattern-recognition receptors (PRR) and resistance (R) proteins, each triggering defense pathways of PRR-triggered immunity and effector-triggered immunity that lead to the induction of defense-related products (Boller and Felix 2009). Plant PRR consist of cell membrane-localized receptor-like kinases (RLK) and supporting receptor-like proteins that can detect pathogen structural molecules (pathogen-associated and microbe-associated molecular patterns) such as fungal chitin and bacterial flagellin (Couto and Zipfel 2016; Jones and Dangl 2006). Pattern recognition receptor-triggered immunity limits the growth of most pathogens, though adapted pathogens may evade the surveillance of PRR with evolved effector proteins and facilitate successful establishment (Couto and Zipfel

2016). The other group of recognition receptors, called R proteins, can identify effector proteins and trigger defense responses. Studies in various plant–pathogen systems have identified R proteins as a key contributor to pathogen resistance (Hammond-Kosack and Parker 2003). Most R proteins contain two conserved domains, a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain (Christie et al. 2016).

The signal cascade required to respond to a pathogen is activated by PRR and R protein receptor recognition. An oxidative burst is triggered within seconds, which induces a defense response by interacting with other signaling molecules such as nitric oxide (NO) and salicylic acid (SA) (Torres et al. 2006). Another signaling event is marked by an increase of cytosolic calcium (Ca²⁺) in plant cells in response to pathogen effectors (Lecourieux et al. 2006), as observed when *Arabidopsis thaliana* was challenged by effector proteins *avrRpm1* and *avrB* of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Grant et al. 2000). Ca²⁺ and the Ca²⁺-sensor protein calmodulin (CaM) interact with NO and H₂O₂, as well as regulate the SA-mediated pathway, which is an important phytohormone signaling pathway that directs plant defense against biotrophic pathogens such as rust fungi (Crampton et al. 2009; Poovaiah et al. 2013). A mitogen-activated protein kinase (MAPK) cascade is then initiated, which phosphorylates target proteins, including WRKY transcriptional regulators, which play a pivotal role in plant defense (Eulgem and Somssich 2007; Meng and Zhang 2013). During defense-response signaling, interplay between phytohormones such as SA, jasmonic acid, and ethylene assists in amplifying the signals from the MAPK cascade (Naidoo et al. 2014). Through different ratios of these phytohormones, sets of transcriptional regulators are induced which, in turn, initiate the production of specific phytoalexins and pathogenesis-related (PR) proteins (Naidoo et al. 2014; Zhao et al. 2005). The PR protein superfamily contains 17 families of unrelated proteins such as chitinases and thaumatin-like proteins (PR5), which have been suggested to target and rupture the structures of pathogens, including chitin and membranes (Sels et al.

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2008). In *Arabidopsis*, for example, increased SA concentrations trigger the coregulatory NPR1 protein, which interacts with basic leucine zipper (bZIP) transcriptional regulators to activate the production of PR protein 1 (Alves et al. 2013). In addition, bZIP factors have shown to be differentially expressed in *Glycine max* (L.) Merr. in response to soybean rust (Alves et al. 2015).

Since 2010, many species of the family Myrtaceae in Australia have been exposed to the exotic pathogen myrtle rust (*Austropuccinia psidii* (G. Winter) Beenken comb. nov., basionym: *Puccinia psidii*) (Beenken 2017; Carnegie and Cooper 2011). Most Australian forests and woodlands are dominated by Myrtaceae family members and the arrival of *A. psidii* in Australia has the potential to cause serious ecological and economic consequences (Pegg et al. 2014). *A. psidii*, which originated from South and Central America, is a biotrophic fungal pathogen that causes lesions on young leaves, flowers, and fruit, resulting in stunted plant growth (Glen et al. 2007) and potential death when the infection is severe (Pegg et al. 2014). It has spread globally, including to Hawaii (Uchida et al. 2006), Florida (Rayachhetry et al. 1997), California (Marlatt and Kimbrough 1979), China (Zhuang and Wei 2011), Japan (Kawanishi et al. 2009), Southeast Asia (McTaggart et al. 2016), and South Africa (Roux et al. 2013). Detected in April 2010 in New South Wales, *A. psidii* has since spread along the east coast of Australia (Carnegie et al. 2010). The pathogen has a wide host range within the family Myrtaceae (Carnegie and Lidbetter 2011). *Melaleuca quinquenervia* (broadleaf paperbark) is a foundation species in coastal wetlands. As a foundation species that is ecologically significant yet highly susceptible to *A. psidii* (Carnegie and Cooper 2011; Pegg et al. 2014), drastic reduction and dieback of *M. quinquenervia* may hinder forest regeneration and disrupt wetland biodiversity (Pegg et al. 2014).

Inoculation tests and field observations suggest that the vast majority of Australian Myrtaceae species are susceptible to *A. psidii* (Giblin and Carnegie 2014; Morin et al. 2012). However, some species show variable symptoms (Morin et al. 2012; Pegg et al. 2014). This suggests that, despite the Australian species being naïve to *A. psidii*, there may be mechanisms of defense that, if identified, might allow some species to persist when challenged by the pathogen (Tobias et al. 2016). This raises several questions, including (i) why do species that have not coevolved with *A. psidii* have some individuals

that were visually symptomless after inoculation and (ii) why does *A. psidii* have such a broad host range?

Woody plants, unlike most herbaceous plants, are long lived and, thus, experience more frequent abiotic and biotic challenges. This promotes the expansion of genes dedicated to pathogen recognition, such as those encoding NBS-LRR resistance proteins. In *Eucalyptus grandis*, which is one of the most studied species in the Myrtaceae family, this large gene family is highly diversified due to events such as tandem duplication (Christie et al. 2016). The reservoir of *R* genes may serve to detect potential pathogens for woody perennials (Tobias et al. 2016). In previous studies, a major resistance locus, *P. psidii* resistance gene 1 (*Ppr1*) was identified in *E. grandis*, a native Australian species widely planted in Brazil (Junghans et al. 2003). *E. grandis* shows variable resistance to *A. psidii* (Ferreira and Silva 1982) and *Ppr1* serves as a marker for identifying disease-resistant cultivars. However, the best resolution of the *Ppr1* locus is >5 centimorgans (Junghans et al. 2003). Although *R* proteins certainly play an important role in pathogen recognition, a range of other genes is also essential in plant defense to orchestrate a complex signaling network and restrict pathogen growth (Naidoo et al. 2014). Further study showed that, within the *E. grandis* *Ppr1* locus, both NBS-LRR genes and bZIP factors were present, suggesting that these bZIP proteins may also contribute to the resistance to *A. psidii* (Thumma et al. 2013).

The specific aim of this study was to use RNA-Seq transcriptome profiling (Wang et al. 2009) to identify the molecular basis of resistance of *M. quinquenervia* to *A. psidii*. This study of *M. quinquenervia* may help elucidate the mechanism of defense in other Australian Myrtaceae species against *A. psidii*, as well as assisting the selection of candidate molecular markers, which can be implemented in breeding resistant plants.

MATERIALS AND METHODS

Plant material. Seventy-four *M. quinquenervia* plants were grown from seed purchased from the Australian Tree Seed Centre (CSIRO, Canberra, Australia). The seed were collected from 10 provenances in Queensland and New South Wales (Fig. 1), with sites ranging from 13°44'S to 31°30'S (latitude), 143°11'E to 153°26'E (longitude), and 1 to 500 m in altitude. Specific site

Fig. 1. Geographic location of collection sites for *Melaleuca quinquenervia* samples. Gray circles and corresponding text labels indicate specific geographical sites of collection along east coast Australia.

locations were Teddington (25°31'S, 152°43'E, 37 m), Dohles Rocks (27°16'S, 153°01'E, 20 m), Bribe Island (27°04'S, 153°11'E, 10 m), Graceville (27°32'S, 153°01'E, 30 m), South of Port Macquarie (31°30'S, 152°40'E, 1 m), Rokeby National Park (13°44'S, 143°19'E, 500 m), Tozer's Gap (12°43'S, 143°11'E, 120 m), 78 km northeast of Gympie Bypass (25°47'S, 152°50'E, 40 m), East Coast of Moreton Island (27°05'S, 153°26'E, 5 m), and Caloundra (26°48'S, 152°59'E, 90 m). The geographic map indicating collection sites was produced using SimpleMappr (Shorthouse 2010) and location names were labeled accordingly.

Inoculation. Pruned plants were grown under controlled conditions at the Australian National University Plant Culture Facility for 3 weeks to ensure that fresh growth was available for inoculation. The plants were then transferred to the Plant Breeding Institute (PBI), University of Sydney, Cobbitty, NSW, Australia. Plants were allowed to acclimatize for a week in the greenhouse and then inoculated with *A. psidii* urediniospores following the methods described by Sandhu and Park (2013). In short, a reference culture (PBI rust culture number 622) was increased from a single pustule raised from the rust sample collected from an *Agonis flexuosa* tree from Leonay, NSW, Australia. Rust inoculum was increased on highly susceptible *Syzygium jambos* (Carnegie and Lidbetter 2011). Young *S. jambos* plants with fresh leaves were included as susceptible controls during inoculations. Rust suspension (2 mg of urediniospores per 1.0 ml of light mineral oil) (Univar Solvent L naphtha 100; Univar Australia Pty. Ltd.) was atomized on the adaxial and abaxial leaf surfaces using an airbrush attached to a motorized compressor. The inoculation room was kept closed for 5 min to allow urediniospores to settle on the leaves. Plants were then moved to a dark room and incubated at 20°C for 24 h in cabinets fitted with misters to maintain >95% relative humidity. After incubation, plants were moved to naturally lit microclimate rooms running at 22 ± 2°C. Inoculations were performed twice on the same plants to test for consistent phenotypes on 13 March 2013 and 11 April 2013.

Disease scoring. Pustules were typically visible 7 days post-inoculation (dpi) (Sandhu and Park 2013). Final scoring was done at 14 dpi, when pustules had fully developed. The plants were then scored based on methods as described by Sandhu and Park (2013) (summarized in Table 1). The six categories of host response scale—highly resistant (HR), resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S), and highly susceptible (HS)—originally suggested by Sandhu and Park (2013) were adjusted for the purpose of this study.

Sampling of leaf tissue. Between four and five leaves (approximately 400 mg) of new growth from each *M. quinquenervia* plant was collected 1 day before inoculation and 5 dpi. The time period 5 dpi was chosen based on the inoculation evaluation of *E. urophylla* to *Austropuccinia psidii* (Alves et al. 2011), where flecks on leaves of susceptible plants were visible and covered 2% of the leaf surface, on average, after inoculation at 4 dpi (Alves et al. 2011).

The leaf samples were then collected, snap frozen in liquid nitrogen, and later stored at -80°C. Based on scoring of the response of *M. quinquenervia* (Table 1), we selected four *M. quinquenervia* individuals for transcriptome assembly to encompass a range of susceptibility levels, from HR to MR, S, and HS. For each individual, we had samples of leaf from before and after inoculation. To address biological replicates for this study, we further selected three HR and three HS plants (the two extremes of resistance and susceptibility), each sampled before and after inoculation, to evaluate gene expressions. Twenty samples were collected for subsequent RNA extraction.

RNA extraction. RNA was extracted using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) after grinding approximately 100 mg of leaf tissue to fine powder in liquid nitrogen with mortar and pestle. We modified the standard protocol by adding 108 mg of sodium-isoascorbate (NA-iASC) or 50 µl of 20% polyvinylpyrrolidone (PVP) into the lysis buffer to optimize RNA quantity (Padovan et al. 2013).

Due to difficulties in obtaining high quantities and qualities of RNA from some samples, we used the Plant RNeasy Plant Mini Kit

(Qiagen) for some extractions. To 100 mg of ground leaf sample, 108 mg of NA-iASC or 50 µl of 20% PVP was added to the lysis buffer to optimize RNA quantity (Padovan et al. 2013).

RNA yield and quality were assessed using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis. From Nanodrop readings, the samples yielded total RNA at 90.96 to 290.01 ng/µl, and had values for absorbance at 260 or 280 nm from 1.7 to 2.01. Gel electrophoresis was used to visually assess RNA quality in each sample, and clear bands of 18S and 28S ribosomal RNA were visible, indicating the high integrity of the RNA samples.

Library preparation and RNA-Seq transcriptome profiling. RNA-Seq libraries were made from total RNA using the TruSeq RNA Sample Preparation Kit (v2; Illumina). We followed the low-sample protocol using 4 µg of total RNA. The adapter-bound DNA fragments were amplified by 10 cycles of polymerase chain reaction (PCR) using Illumina PCR Primer Cocktail. Libraries were quantified with a Qubit fluorometer (Life Technologies) and equimolar amounts from each library were pooled. The pooled library was sent to the Biomolecular Resource Facility at the Australian National University for HiSeq2500 sequencing (Illumina) with a 2-by-150-bp protocol. RNA-Seq data were deposited into the Sequence Read Archive (SRA) database under SRA identifier SRP095052 and BioProject accession number PRJNA357284 (public access will be available after article publication).

De novo transcriptome assembly. Raw reads produced from the HiSeq2500 platform (Illumina) were first deconvoluted. We developed a pipeline for transcriptome assembly and differential gene expression analysis. Read quality was assessed using the FastQC program (Andrews 2010), and low-quality reads (mean Phred score < 20) were trimmed using Trimmomatic (version 0.3) (Bolger et al. 2014). The threshold values for trimming were LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 5:30, and MINLEN: 60. Trinity (Grabherr et al. 2011) was used to assemble the *M. quinquenervia* reference transcriptome de novo. All eight samples encompassing the range of susceptibility (HS, S, R, and HR, each sampled twice) were used simultaneously for the assembly. To remove redundancy from transcripts in the transcriptome, CD-HIT-EST (Li and Godzik 2006) was used at a threshold of 0.94 identity. This threshold was set based on a transcriptome study of *M. alternifolia* (Bustos-Segura et al. 2017), where 0.94 identity was optimal to remove potential allelic variants while keeping genes of interest; the threshold differentiated the two terpene synthase genes of high similarity (pairwise nucleotide identities of 96.6%).

Validation of transcriptome assembly quality. The Core Eukaryotic Genes Mapping Approach (CEGMA) package (version 2.4) (Parra et al. 2007) was used to determine the completeness of the assembled transcriptome. It utilizes a set of 248 extremely conserved core eukaryotic genes (CEG) to evaluate the number of essential functional orthologs covered in the assembly (Parra et al. 2009). The package has been used to validate transcriptomes in other studies, including gene expression of plants in response to pathogen infection (Kovi et al. 2016).

TABLE 1. Scoring scale for measuring *Melaleuca quinquenervia* host response against *Austropuccinia psidii*

Infection symptoms	Host response
No visible sign of infection	Highly resistant (HR)
Mild hypersensitivity/necrosis/flecks/dark flecks	Resistant (R)
Restricted pustule/dark gray surrounding/ chlorosis/necrosis	Moderately Resistant (MR)
Small to medium pustules (low density)/ chlorosis	Moderately susceptible (MS)
Fully developed pustules (medium to high density)	Susceptible (S)
Fully developed pustules (high density)	Highly susceptible (HS)

Transcriptome functional protein annotation. The transcriptome was first annotated using the Trinotate annotation pipeline (Haas et al. 2013). The pipeline annotated transcriptome sequences by BLASTX search (Camacho et al. 2009) to the nonredundant protein database, UniProtKB/Swiss-Prot (UniProt Consortium 2014). TransDecoder (Haas et al. 2013) was then used to identify the longest open reading frame (ORF) peptide candidates from the transcriptome, and candidates were further annotated by BLASTP search against databases, including UniProtKB/Swiss-Prot, Protein Families Database (Pfam), Evolutionary Genealogy of Genes: Nonsupervised Orthologous Groups, and Enzyme Commission, which assisted in the identification of the putative biological process, molecular function, and cellular localization of the transcripts. An *E*-value cut-off of 0.1 was used for annotations. Transcripts that were fungal derived were determined by annotations to the UniProtKB/Swiss-Prot database; transcripts that had the classification “fungi” were classified as fungal-derived. In addition, we annotated the transcriptome by TBLASTX search (*E*-value cut-off of 0.1) to the *Arabidopsis thaliana* genome (TAIR10) from The *Arabidopsis* Information Resource (TAIR) to acquire gene ontology (GO) annotation for the transcripts, because *Arabidopsis* is the most comprehensively annotated plant genome. A candidate transcript of interest (Mq47801_c1_seq8) was further manually assessed for homology by performing BLASTP using the longest ORF sequence obtained from TransDecoder against the National Center for Biotechnology Information nonredundant protein database. Subsequently, the trimmed reads of HR and HS plants of four biological replicates each were mapped against the transcriptome using Bowtie 2 (Langmead et al. 2012). The mapped reads were then used for building the count matrix, which was generated by featureCounts (Liao et al. 2014). Using featureCounts, the number of uniquely mapped reads were counted for each annotated transcript (GTF file format), and the count matrix was used for differential gene expression analysis.

Differential gene expression analysis. We compared differential gene expression in four groups: (i) before and after infection within the HS phenotype (HS versus HS_rust), (ii) before and after infection within the HR phenotype (HR versus HR_rust), (iii) before infection between HS and HR phenotypes (HS versus HR), and (iv) after infection between HS and HR phenotypes (HS_rust versus HR_rust).

Two differential expression (DE) analysis packages, DESeq2 (Love et al. 2014) and edgeR (Robinson et al. 2010), were used to distinguish differentially expressed genes (DEG). Using two DE analysis packages of differing algorithms helps to filter out bias from sample size and sequencing depth in the DEG counts (Robles et al. 2012). The raw count matrix derived from featureCounts was used in DESeq2 and edgeR; all packages are available from the Bioconductor project in R.

For settings in DESeq2, normalization factors were calculated for each library of varying sequencing depths using the means-of-ratios method (Anders et al. 2013). The estimation of gene-wise dispersion was done by maximum likelihood and shrinkage toward the fitted parametric curve, which outputs maximum a posteriori as the final estimate of the dispersion (Love et al. 2014). Tests for DEG of adjusted *P* value < 0.05 was performed by the Wald test for significance of coefficients on the negative binomial generalized linear model (GLM) (Love et al. 2014).

For edgeR, normalization factors were calculated using the trimmed mean of M values (TMM) method (Robinson and Oshlack 2010). M values are defined as the log ratio of level counts for each gene between two samples. The TMM normalization in edgeR returned normalized factors, the counts per million (CPM). Common, trended, and tagwise dispersions (using the Cox-Reid profile-adjusted likelihood [CR] method) were estimated, and negative binomial GLM likelihood ratio tests were performed to detect significantly DEG (McCarthy et al. 2012). The log₂CPM threshold value was ≥1. Threshold values were set and genes were considered as

significantly differentially expressed by *P* value and false discovery rate (FDR) < 0.05 (Benjamini and Hochberg 1995). Consensus DEG detected by both DE packages were visualized by Venn diagrams using Venny (version 2.1.0) (Oliveros 2007), and the DEG were then used for downstream analyses. Subsequent analyses used fungi and vascular plant (*Tracheophyta* spp.)-annotated transcripts.

To compare the number of unique and common DEG present in the four groups (HS versus HS_rust, HR versus HR_rust, HS versus HR, and HS_rust versus HR_rust), a four-set Venn diagram was created using Venny (version 2.1.0) (Oliveros 2007). Hierarchical clustering heatmaps of DE test groups HS versus HR and HS_rust versus HR_rust were produced using the *heatmap.2* function in ‘gplots’ from the Bioconductor project in R (R version 3.2.3). The heatmaps were made by a measure of Euclidean distance, the default option of *heatmap.2*. Normalized counts produced from the normalization method of transcript per kilobase million (TPM) were used to generate the heatmaps.

Volcano plots of DE test groups HS versus HR, HS_rust versus HR_rust, and HS_rust versus HR_rust, excluding transcripts annotated to fungi orthologs in the UniProtKB/Swiss-Prot database, were created using the *plot()* function in R (R version 3.2.3). NormCPM trimmed (log₂CPM threshold value ≥ 1) data were used as input, and each point indicated a transcript. The cut-off value for significant differentially expressed transcripts was FDR < 0.05, and a gray line across the plot showed dark gray points above the line as significantly expressed. For transcripts of an absolute logFC value > 6, light gray points were applied. Selected text labels were adjusted from labels created using the *textxy* function in the ‘calibrate’ package (R version 3.2.3).

Data were depicted in MapMan. To do so, we used the DEG identified in each of the four comparisons (HS versus HR, HS_rust versus HR_rust, HS versus HS_rust, and HR versus HR_rust), extracted *A. thaliana* gene identifier (AT_ID), and calculated log fold change between the comparisons. The lists were then checked for multiple occurrences of AT_ID. In most cases where two or more transcripts had the same AT_ID, the fold change values were highly similar and all but one (the largest fold change) were deleted. In the few cases where the fold change was in opposing directions, the AT_ID was deleted. This can easily be explained for some secondary metabolism genes, where one metabolite synthesis gene is induced, while another is reduced in expression yet both have the same AT_ID as closest homolog. Data were then imported into MapMan v3.1.1 and the ‘Metabolism overview’, ‘Secondary metabolism’, and ‘Biotic stress’ mappings were chosen to depict the comparisons.

GO enrichment analysis. Following the differential gene expression analysis described above, gene enrichment analysis was performed by AgriGO (Du et al. 2010). Two DE test groups which identified DEG within each phenotype in response to *Austropuccinia psidii* (HS versus HS_rust and HR versus HR_rust) were enriched separately using AgriGO and compared for differences in overrepresented GO terms. For the enrichment, transcripts that were annotated to both vascular plant (*Tracheophyta* spp.) transcripts in UniProtKB/Swiss-Prot and to TAIR10 were selected, and the TAIR10 database was used as the reference background. The statistical parameters used were Fisher’s statistical method and the Yekutieli (FDR under dependency) multitest adjustment method (Benjamini and Yekutieli 2001), with a cut-off value at the significance level of 0.01 and a minimum number of mapping entries of 5. Thirty-eight significantly enriched GO biological process terms were selected for visualization, and the y-axis represents $-\log_{10}(\text{adjusted } P \text{ value})$ of GO enrichment.

Model of defense responses in *M. quinquenervia* against *A. psidii* infection. Defense-related transcripts were extracted from the transcriptome data of *M. quinquenervia*, which was annotated against databases UniProtKB/Swiss-Prot, TAIR10, and Pfam. The number of significantly upregulated transcripts was compared between HS and HR. The column labeled ‘Genes’ represented the total number of the corresponding gene type identified in *M. quinquenervia* transcriptome, and the percentage under each phenotype (HS and HR) represented the percentage of genes that was significantly upregulated

TABLE 2. Summary of de novo assembly for *Melaleuca quinquenervia* transcriptome^a

Assembly	Reads
RNA-Seq data for assembly	
Total number of reads	103,521,497
Read length	151
Total number of reads after filtering by quality	70,666,254
Reads used for assembly (%)	68.3
Trinity de novo transcriptome assembly	
Total transcripts	192,557
N50 (bp)	2,426
GC (%)	43.45
Minimum transcript length (bp)	201
Maximum transcript length (bp)	16,047
Median transcript length (bp)	1,083
Average transcript length (bp)	1,474
Total assembled bases	283,846,943
Nonredundant transcripts	138,249
Reads of HS and HR individuals mapped to the transcriptome	
Total reads	259,963,239
Total reads mapped	255,847,624
Mapping rate (%)	98.7

^a HS = highly susceptible and HR = highly resistant. Reads from four biologically independent individuals of HS and HR phenotypes before and after infection were mapped.

TABLE 3. Validation of de novo transcriptome assembly using Core Eukaryotic Genes Mapping Approach analysis^a

Analysis	<i>Melaleuca quinquenervia</i> transcriptome
Complete ultra-conserved CEG present (%)	99.19
Average number of orthologs per CEG	3.11
Observed CEG with > 1 orthologs (%)	76.83

^a CEG = core eukaryotic genes, 248 in total.

after *A. psidii* infection. The genes were grouped according to biological processes during defense (Azaiez et al. 2009; Meyer et al. 2016; Naidoo et al. 2014; Oates et al. 2015). Due to the rapid evolution of terpene synthase genes, we utilized a different annotation strategy for this gene family. We searched our assembled transcriptome for two conserved domains in Pfam, the N-terminal (PF01397) and the C-terminal (PF03936) domains.

Selected DEG displaying variable expression among biologically independent samples. To investigate variations in gene expression among biologically independent samples of HS and HR phenotypes, several candidate DEG were selected for further study, including recognition factors NBS-LRR and RLK, and defense-related factors such as PR proteins. Fungal-derived genes were also selected to show expression differences of these genes among HS and HR sample individuals. Normalized counts derived from normalization method TPM were used for comparing relative abundances between individuals. Clustered bar graphs were produced to illustrate gene expression differences across all individuals before and after infection.

De novo assembly of the *A. psidii* genome and mapping of *A. psidii*-inoculated samples. Raw reads of *A. psidii* (PBI accession number 115012-Mr) produced by Tan and colleagues (2014) were exported from the SRA (BioProject Accession: SRX903408) for de novo assembly. We assembled the reads using the CLC Genomics Workbench (CLC Bio) after trimming by quality. TopHat (version 2.1.0) (Trapnell et al. 2009) was then used to map the reads of inoculated *M. quinquenervia* leaf samples against the assembled *A. psidii* genome.

RESULTS

Scoring *M. quinquenervia* plants for myrtle rust resistance. Seventy-four *M. quinquenervia* plants were grown from seed collected along the coast of Queensland and northern New South Wales, Australia (Fig. 1). When newly developing

Fig. 2. Relatedness among biologically independent individuals of highly susceptible (HS) and highly resistant (HR) plants before and after *Austropuccinia psidii* infection using multidimensional scaling plot with pairwise comparison in edgeR. Suffix ‘_rust’ represents the sample after infection. The x-axis and y-axis represent all expression levels of plant-derived transcripts in replicates and conditions. Dashed and solid vectors specify HS and HR sample changes after infection, respectively, with circled regions showing the overall trend of change in HS and HR plants, respectively.

leaves were present, they were inoculated and scored for myrtle rust resistance (Table 1). Each plant was inoculated twice and nine individuals received scores that differed by more than one point of the scale (e.g., from HR to HS) between the two inoculations (Supplementary Table S1). These plants were excluded from further experiments. Rust screening and scoring at 14 days postinoculation (dpi) revealed that the majority of plants (60%) are susceptible to *A. psidii* (MS, S, and HS). Of the plants which showed varying degrees of resistance to *A. psidii* (MR, R, and HR), two-thirds were HR plants, which were completely immune, with no visible symptoms (Supplementary Fig. S1).

Transcriptome sequencing, de novo assembly, and mapping. mRNA from leaves sampled before and at 5 dpi were sequenced, because previous studies of *A. psidii* have reported susceptible *E. urophylla* showing flecks at 4 dpi. To encompass a range of susceptibility levels for transcriptome construction, HS, S, MR, and HR *M. quinquenervia* plants were selected for assembly. Paired-end reads were obtained using an Illumina HiSeq2500 platform and, in total, 70 million reads were available after filtering by quality (Table 2). We assembled 192,557 transcripts using Trinity, with transcripts representing putative isoforms. After removing redundancy using CD-HIT-EST, 138,249 nonredundant transcripts were used for mapping raw reads of each sample and differential gene expression analyses (Table 2). In addition, we assessed the

completeness of the improved transcriptome by the CEGMA analysis (Table 3). The percentage of complete ultraconserved CEG present in the assembled transcriptome was 99.19% (Table 3). The transcriptome had 42% of the transcripts annotated to the nonredundant protein sequence database UniProtKB/Swiss-Prot, and 30% had blast hits to the *Arabidopsis* TAIR10 database.

For downstream differential gene expression analyses, HS and HR plants were chosen to represent the two extremes of susceptibility and resistance in response to *A. psidii* infection. In total, 259 million filtered reads from HS and HR plants of four biological replicates each, both before and after infection, were mapped against the transcriptome using Bowtie 2. Reads from the eight individuals resulted in 255 million mapped reads in total, with an average mapping rate of 98.7% (Table 2; Supplementary Table S2).

Global view of DEG in resistant and susceptible *M. quinquenervia* plants before and after *A. psidii* infection. Relatedness of gene expression patterns among HS and HR samples before and after *A. psidii* infection was evaluated by multidimensional scaling (Fig. 2). HS plants were more similar to each other in their changes in expression after *A. psidii* infection than to HR plants' changes in expression after the infection. Notably, HR2 and HR3 did not vary in expression as much as all other samples after they were each infected with *A. psidii* (Fig. 2).

Fig. 3. Bar graphs and Venn diagram summarizing significantly differentially expressed genes (DEG) comparing resistant to susceptible samples both before and after rust infection. HS = highly susceptible, HR = highly resistant, and _rust indicates the sample after infection. **A**, Total DEG expressed in each group; **B**, fungal-derived DEG; **C**, plant-derived DEG; and **D**, the number of common and unique plant-derived DEG identified in each group (5% false discovery rate). Light gray bars in A, B, and C show DEG that were overexpressed in the second term of each comparison (fold-change [FC] > 1) and dark gray bars show the number of DEG downregulated (FC < 1).

Fig. 4. Heatmaps of plant-derived significantly differentially expressed genes (DEG) between highly susceptible (HS) and highly resistant (HR) *Melaleuca quinquenervia* phenotypes **A**, before and **B**, after *Austropuccinia psidii* infection. Each phenotype has four biologically individual samples (1 to 4). The y-axis represents each DEG expressed across samples. In all, 151 DEG were observed in A and 1,136 DEG were observed in B. The shading scale indicates the gene expression value ($\log_2$ -transformed transcripts per kilobase million).

Fig. 5. Volcano plot of differentially expressed genes (DEG) between highly susceptible (HS) and highly resistant (HR) plants **A**, before infection; **B**, at 5 days postinoculation (dpi); and **C**, at 5 dpi of plant-only transcripts. Points above the gray line are significantly DEG (5% false discovery rate). Positive logFC indicates higher gene expression in HR while negative logFC indicates higher gene expression in HS. Light gray points indicate $|\log FC| > 6$. Abbreviations: SULTR = Sulfate transporter, TPS = terpene synthase, CESA = cellulose synthase, CHS = chalcone synthase, CRK = cysteine-rich receptor-like kinase, RLP = receptor-like protein, ALDH = aldehyde dehydrogenase, LRR-RLK = leucine-rich repeat-receptor-like kinase, WAK = wall-associated kinase, AGO = argonaute protein, LecRK = lectin receptor kinase, NBS-LRR = nucleotide-binding site leucine-rich repeat protein, PAL = phenylalanine ammonia-lyase, IMDH = isopropylmalate dehydrogenase, PLT = polyol transporter, FTHFS = formyltetrahydrofolate synthetase, GAD = glutamate decarboxylase, MnSOD = manganese superoxide dismutase, and PRX2B = peroxiredoxin-2B.

We then assessed DEG in four interaction groups of HS and HR, before inoculation and 5 dpi with urediniospores of *A. psidii* (Fig. 3). We used consensus DEG detected by two differential expression analysis packages, DESeq2 and edgeR (Supplementary Fig. S2). A relatively small difference in expression was observed between HS and HR prior to rust infection (Fig. 3A). However, we were surprised to find that, at 5 dpi, HS exhibited the greatest number of DEG (Fig. 3A). Therefore, we extracted fungal-annotated transcripts from the total DEG to investigate the presence of fungal transcripts. This showed that 16% of DEG in HS (5 dpi) were fungal-derived (Fig. 3B). After excluding fungal transcripts, HS still showed approximately twice as many plant-derived DEG compared with HR after infection (Fig. 3C and D). In addition, the number of fungal-derived DEG in HS (5 dpi) was 12 times greater than the number of fungal-derived DEG in HR (5 dpi) (Fig. 3B).

Heatmaps of plant-derived DEG showed a clear separation between HS and HR samples both before and after infection (Fig. 4). However, the expression abundance of each gene among biologically independent samples was variable, particularly for HR, which contained defense-related gene candidates (Fig. 4). We then visualized fold changes of total and plant-only DEG using volcano plots (Fig. 5). Among the more significantly overexpressed transcripts in HR compared with HS were many RLK and NBS-LRR proteins (Fig. 5A and C). We also found that, among fungal-derived transcripts overexpressed in HS compared with HR at 5 dpi (Fig. 5B), one of the most significantly expressed transcripts encoded a homolog of a fungal cellulase (P07982) (Fig. 5B). A

transcript encoding a homolog of a fungal planta-induced rust protein (O00057) was also highly overexpressed in HS compared with HR at 5 dpi (Fig. 5B).

Additionally, we validated the abundance of *A. psidii* in HS and HR by mapping samples to a draft *A. psidii* genome sequence (Tan et al. 2014) (Supplementary Table S3). In HS (5 dpi) samples, 2% of the reads on average mapped to *A. psidii*, whereas HR (5 dpi) had 0% of reads mapped for all samples (Supplementary Fig. S3).

GO enrichment. To identify compositional differences of the changes in gene expression in HS and HR plants in their response to *A. psidii* infection at 5 dpi, we performed GO enrichment on plant-derived DEG in interaction groups HS versus HS_rust and HR versus HR_rust (Fig. 6). Contrary to what we expected, we found more defense-related GO terms enriched in HS (5 dpi) than in HR (5 dpi) (Fig. 6; Supplementary Table S4). Several GO terms enriched only in HS (5 dpi) included ‘response to wounding’, ‘response to oxidative stress’, ‘jasmonic acid metabolic process’, ‘regulation of plant-type hypersensitive response’, and others (Fig. 6). On the other hand, HR (5 dpi) contained GO terms that were uniquely enriched, including ‘microtubule cytoskeleton organization’, ‘actin cytoskeleton organization’, ‘response to gibberellin stimulus’, and others (Fig. 6).

Model of defense in *M. quinquenervia* in response to *A. psidii*. Specific defense-related genes from DEG were further explored in HS and HR plants in their response to *A. psidii* infection at 5 dpi (Fig. 7). Similar to results of the enrichment analysis, HS (5 dpi) overall had more defense-related genes induced compared

Fig. 6. Selected gene ontology (GO) terms which were significantly enriched within highly susceptible (HS) phenotype (black bar) and highly resistant (HR) phenotype (gray bar) in response to *Austropuccinia psidii* infection at 5 days postinoculation. The y-axis represents $-\log_{10}(\text{adjusted } P \text{ value})$ of GO enrichment.

with HR (5 dpi) (Fig. 7). Only one transcript (<1%) encoding an NBS-LRR protein was significantly induced in HR at 5 dpi. The transcript (Mq47801_c1_seq8) encoded a homolog of *E. grandis Tobacco mosaic virus* resistance protein N (identity score = 72% and *E*-value = 1×10^{-86}) (XP_018726807.1) (Fig. 7). However, HR had more WRKY transcriptional regulators, chitin-binding proteins, and osmotin and thaumatin-like proteins (PR-5) induced at 5 dpi compared with HS (Fig. 7). Of particular note, HR had more expansins induced at 5 dpi than HS (Fig. 7).

Variation in expression of candidate genes among resistant individuals. In total, 14 and 17 RLK were observed to be overexpressed in HR compared with HS before and after infection, respectively (Table 4 shows selected RLK transcripts; the full list is detailed in Supplementary Table S5). Of those, five were overexpressed both before and after infection. In all, 2 and 10 NBS-LRR class protein-encoding transcripts were overexpressed in HR in comparison with HS before and after infection (Table 4). In particular, a transcript (Mq53985_c0_seq5) encoding a homolog of *Arabidopsis* coiled-coil class NBS-LRR protein was overexpressed both before and after infection (Table 4).

Among downstream defense-related genes, we observed two oxidative burst-detoxifying factor glutathione S-transferases (GST) overexpressed in HR compared with HS at 5 dpi (Table 4). A

number of transcripts encoding homologs of *Arabidopsis* NAC (NAM, ATAF, and CUC), bHLH, MADS-box, and WRKY transcriptional regulators were overexpressed in HR (Table 4). In addition, cutin and lignin biosynthesis-related genes were overexpressed in HR, such as white-brown complex homolog protein 11 and HXXXD-type acyl-transferase family proteins (Table 4). Several PR proteins belonging to a range of subclasses (PR-4, PR-5, PR-9, and PR-10) were significantly overexpressed in HR compared with HS at 5 dpi (Table 4).

M. quinquenervia is genetically diverse and the plants were sourced from several provenances (Fig. 1). Not surprisingly, we found strikingly large differences in expression of defense-related gene candidates among resistant individuals. For example, we observed an LRR-RLK gene (Mq47245_c0_seq3), although identified by DE packages as significantly overexpressed in HR compared with HS (Table 4), that was, in fact, only greatly induced in resistant samples 1 and 4 but not in 2 and 3 (Fig. 8A). Similar examples include a cysteine-rich kinase (Mq42098_c0_seq1) and an NBS-LRR protein (Mq52241_c2_seq1) that were only highly induced in sample 2 (Fig. 8B and C; Table 4), as well as GST (Mq41846_c0_seq3), WRKY transcriptional regulator (Mq47352_c0_seq2), PR-1 (Mq47510_c1_seq1), and PR-5 (Mq44909_c0_seq3) (Fig. 8; Table 4). Other examples, including PR-2 (Mq44699_c0_seq1), PR-3 (Mq36473_c0_seq1), and PR-4 (Mq50812_c0_seq1), are shown in Supplementary

Fig. 7. Hypothetical model of defense-related factors upregulated in highly susceptible (HS) and highly resistant (HR) *Melaleuca quinquenervia* plants in response to *Austropuccinia psidii* infection at 5 days postinoculation. The column labeled 'Genes' represents the total number of transcripts of the corresponding gene observed to be expressed in the *M. quinquenervia* transcriptome, and the percentage under columns labeled HS and HR represents the percentage of transcripts that was significantly upregulated (fold-change > 1) after *A. psidii* infection. Abbreviations: MAPKKK = mitogen-activated protein kinase kinase kinase, MAPKK = mitogen-activated protein kinase kinase, MAPK = mitogen-activated protein kinase, CDPK = calcium-dependent protein kinase, GST = glutathione S-transferase, bZIP = basic region/leucine zipper motif, bHLH = basic helix-loop-helix, MYB = myeloblastosis, DXS = 1-deoxyxylulose-5-phosphate synthase, DXR = 1-deoxyxylulose-5-phosphate reductoisomerase, HMGS = 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR = 3-hydroxy-3-methylglutaryl-CoA reductase, PAL = phenylalanine ammonia-lyase, C4H = cinnamate 4-hydroxylase, F3H = flavanone 3-hydroxylase, CAD = cinnamyl alcohol dehydrogenase, and SAM-MTase = S-adenosyl-L-methionine-dependent methyltransferase. Gray shading indicates which genotype had a higher proportion of genes induced.

Fig. S4. Furthermore, some genes were not detected by DE analyses yet showed a high degree of induction in some resistant individuals. A transcript (Mq51367_c0_seq1) encoding a homolog of *Pinus taeda* phenylalanine ammonia-lyase (PAL) was only detected as an overexpressed DEG in HR compared with HS before infection (Table 4), although the expression of the transcript was more pronounced in resistant samples 1 and 4 after infection (Fig. 8E). Flavanone 3-hydroxylase (F3H) (Mq49092_c0_seq3) was not identified as significantly overexpressed in HR compared with HS but resistant samples 1 and 4 showed a higher expression abundance than that observed in all HS samples (Fig. 8F).

DISCUSSION

In this study, we aimed to identify resistant and susceptible individuals in the ecologically important foundation tree species

M. quinquenervia to the exotic fungal pathogen *A. psidii* and investigate the molecular mechanism of defense using RNA-Seq transcriptome profiling. We found that (i) susceptible *M. quinquenervia* contained high levels of fungal-derived transcripts, (ii) susceptible *M. quinquenervia* had a large number of defense-related genes induced at 5 dpi, and (iii) biologically independent samples of resistant *M. quinquenervia* showed variation in expression of defense-related genes, suggesting that resistant plants may have different modes of effective defense against *A. psidii*.

One of the most noteworthy findings was that susceptible plants had higher levels of fungal-derived transcripts than resistant plants (Figs. 3B and 5B). This may have indicated strong growth of *A. psidii* inside the leaves. Several transcripts associated with haustoria formation were overexpressed in susceptible plants, which implied that *A. psidii* development may have reached the parasitic feeding stage by 5 dpi. For example, one transcript (Mq30159_c0_seq1) encoded a

TABLE 4. Selected defense-related gene candidates of *Austropuccinia psidii* resistance in *Melaleuca quinquenervia*

Transcript ID	UniProtKB /Swiss-Prot	TAIR ^a	Functional annotation description ^b	FC (HR/HS) ^c	FC (HR_r /HS_r) ^d
Pathogen recognition					
Mq53752_c0_seq35	Q9LT96	AT1G79620	LRR-RLK	2.65	–
Mq38788_c0_seq3	Q9LHP4	AT4G28560	RLK 2	–	43.35
Mq49241_c0_seq1	Q9M0X5	AT4G23220	CR RLK	34.44	11.91
Mq42098_c0_seq1	Q9M0X5	AT4G23180	CR RLK	–	28.37
Mq54541_c0_seq3	Q9LXA5	...	L-type lectin RLK	4.15	5.60
Mq51873_c0_seq5	P0C5E2	AT1G66880	Serine/threonine receptor kinase	–	5.34
Mq30776_c0_seq1	Q9SKB2	AT2G31880	LRR receptor-like serine/threonine/tyrosine-protein kinase SOBIR1	–	4.10
Mq47245_c0_seq3	C0LGS2	AT1G47890	LRR receptor-like serine/threonine kinase	27.48	35.07
Mq49053_c0_seq5	Q9LMN7	AT1G21230	WAK family protein	5.53	–
Mq53009_c0_seq2	Q0WNY5	AT4G31110	WAK family protein	5.29	8.95
Mq39830_c0_seq1	Q9LMN8	AT1G16120	WAK family protein	–	7.64
Mq53896_c0_seq10	Q9FID5	AT5G39020	Malectin/RLK family protein	–	3.27
Mq36330_c0_seq1	Q8LPB4	AT3G11010	Phytosulfokine LRR-RLK	–	15.39
Mq54552_c0_seq21	Q9C9H7	AT1G45616	RLP	6.75	–
Mq50189_c1_seq2	Q9C9H7	...	RLP	–	16.95
Mq43862_c0_seq1	Q9C9H7	AT1G71390	RLP	–	13.26
Mq47123_c0_seq6	Q9C9H7	...	RLP	–	10.02
Mq54552_c0_seq2	Q9C9H7	AT3G24900	RLP	3.62	8.83
Mq47032_c0_seq4	Q8L3R3	AT1G12210	Disease resistance protein RFL1	–	4.76
Mq54550_c0_seq4	Q7XA39	...	Putative disease resistance protein RGA4	–	4.06
Mq52241_c2_seq1	Q7XA39	...	Putative disease resistance protein RGA4	76.09	–
Mq53803_c0_seq2	Q9FJB5	...	Disease resistance RPP8-like protein 3	2.65	–
Mq45981_c0_seq5	Q9LRR4	AT3G14470	NB-ARC domain-containing disease resistance protein (putative disease resistance RPPL1)	–	3.61
Mq54082_c0_seq3	O81825	AT4G27190	NB-ARC domain-containing disease resistance protein	–	3.06
Mq53985_c0_seq5	Q9LMP6	AT1G15890	Disease resistance protein (CC-NBS-LRR class)	7.30	5.40
Mq53282_c0_seq3	Q8RXS5	AT5G63020	Disease resistance protein (CC-NBS-LRR class)	–	2.59
Mq52792_c0_seq1	Q40392	AT5G17680	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	–	30.18
Mq53919_c0_seq2	Q40392	AT5G11250	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	–	22.44
Mq36538_c1_seq3	Q40392	AT5G40920	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	–	16.26
Mq46167_c0_seq5	Q40392	AT5G45050	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	2.58	–
Mq50430_c0_seq4	Q40392	AT5G38850	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	–	3.49
Mq54527_c0_seq2	Q40392	AT5G17680	Disease resistance protein (TIR-NBS-LRR class) (TMV resistance protein N)	–	2.33
Mq51888_c0_seq3	Q9FL92	AT1G69550	Disease resistance protein (TIR-NBS-LRR class) (WRKY16 containing TIR-NBS-LRR domain)	–	2.28

(Continued on next page)

^a TAIR = The *Arabidopsis* Information Resource.

^b LRR = leucine-rich repeat, RLK = receptor-like kinase, CR = cysteine-rich, WAK = wall-associated kinase, NB-ARC = nucleotide-binding domain homologous to APAF-1, R proteins, and CED-4, CC-NBS = coiled-coil nucleotide-binding site, TIR = toll interleukin 1 receptor, TMV = *Tobacco mosaic virus*, bHLH = basic helix-loop-helix, 2OG = 2-oxoglutarate, and WBCH = white-brown complex homolog.

^c FC(HR/HS) = fold-change (FC) value of comparing highly resistant (HR) to highly susceptible (HS) before infection.

^d FC value of comparing HR to HS after infection.

homolog of the *Uromyces fabae* planta-induced gene 1, which has been suggested to aid mycelium growth by synthesizing the essential vitamin thiamine, and has been shown to be localized and most highly expressed in haustoria (Sohn et al. 2000). In addition, susceptible plants, on average, had 2% of reads mapped to the *A. psidii* draft genome, compared with 0% for all resistant plants, which further suggested that only susceptible plants allowed the growth of *A. psidii* at 5 dpi.

Susceptible plants also had an abundance of defense-related genes induced at 5 dpi (Figs. 3A, 6, and 7). High levels of defense-related genes observed in susceptible plants suggest overcompensation—susceptible plants may have mounted a defense against the pathogen but this occurred too late. A similar phenomenon was observed in the RNA-Seq study by Meyer and colleagues (2016), which showed that the Myrtaceae species *E. nitens* elicited a range of defense-related factors of secondary metabolite biosynthesis, as well as PR proteins, when infected with *Phytophthora cinnamomi*,

although the plants were susceptible. Similarly, the study by Fung and colleagues (2008) observed that *Vitis vinifera* susceptible to powdery mildew showed 625 induced transcripts after infection whereas, in resistant *V. aestivalis*, only 3 transcripts were induced after infection.

Different individuals of resistant *M. quinquenervia* are likely to possess and mount different modes of defense against *A. psidii*. Although all biologically independent resistant samples showing total immunity were able to successfully defend against *A. psidii* infection, as suggested by <0.1% of the reads being mapped to the *A. psidii* draft genome, the expression levels of defense-related genes among individuals were disparate (Fig. 8). Disparities of levels of gene expression observed among resistant individuals bring into focus a potential challenge in studying differential gene expression in nonmodel, wild-sourced species such as *M. quinquenervia*. We may need to reconsider the use of the conventional workflow of DE analysis that caters largely for clonal materials, because nonmodel

TABLE 4. (Continued from previous page)

Transcript ID	UniProtKB /Swiss-Prot	TAIR ^a	Functional annotation description ^b	FC (HR/HS) ^c	FC (HR_r /HS_r) ^d
Mq54556_c0_seq13	O23530	AT4G08450	Disease resistance protein (TIR-NBS-LRR class) (Protein suppressor of npr1-1, constitutive 1)	–	3.05
Mq52018_c0_seq9	O82500	AT4G11170	Disease resistance protein (TIR-NBS-LRR class)	–	2.31
Oxidative stress-related					
Mq41267_c0_seq2	Q9SU63	AT1G54100	Aldehyde dehydrogenase	3.16	–
Mq52478_c0_seq3	O80763	AT1G60420	Nucleoredoxin	–	3.46
Mq22031_c0_seq1	O80763	AT1G60420	Nucleoredoxin	–	4.61
Mq52478_c0_seq3	O80763	AT1G60420	Nucleoredoxin	1.71	–
Mq50457_c0_seq2	Q8GXJ4	AT1G05200	Glutamate receptor	–	9.13
Mq41846_c0_seq3	P42760	...	Glutathione S-transferase	–	6.35
Mq35636_c0_seq3	Q9FQA3	AT3G43800	Glutathione S-transferase	–	3.14
Transcriptional regulation					
Mq39879_c0_seq1	Q9FLI1	...	bHLH family transcriptional regulator	–	6.14
Mq54350_c0_seq2	Q9SZ67	...	WRKY transcription factor	–	3.32
Mq46661_c0_seq1	Q9SK55	AT2G43000	NAC domain-containing protein	–	5.46
Mq52036_c0_seq7	Q9FIW5	AT1G69490	NAC domain-containing protein	–	3.67
Mq44339_c0_seq1	Q9FIW5	...	NAC domain-containing protein	–	2.86
Mq48248_c0_seq5	Q84K00	AT1G65910	NAC domain containing protein	3.69	–
Mq54283_c0_seq3	Q6EP49	...	MADS-box transcription factor	2.97	–
Phytohormone signaling-related					
Mq45843_c0_seq2	Q39255	AT5G42190	E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 family protein	–	6.95
Mq42162_c0_seq24	P43254	AT2G32950	E3 ubiquitin-protein ligase	–	3.55
Mq33564_c0_seq1	Q9SHY6	AT1G65680	Expansin B2	–	19.37
Mq31345_c0_seq1	Q9C7I1	AT3G05200	RING/U-box superfamily protein	–	7.27
Mq54842_c0_seq1	Q8L5A7	AT5G07010	Sulfotransferase	–	24.67
Mq50091_c1_seq1	P80884	AT1G20850	Xylem cysteine peptidase	–	18.89
Mq50840_c0_seq1	Q67ZU1	...	Triacylglycerol lipase	3.19	–
Mq42138_c0_seq1	Q9LHE3	AT5G10770	Aspartic protease in guard cell	2.57	–
Secondary metabolism-related					
Mq27425_c0_seq1	P93771	AT1G52800	2OG and Fe(II)-dependent oxygenase superfamily protein	7.73	–
Mq43962_c0_seq1	P51094	AT5G17050	UDP-glycosyltransferase superfamily protein	–	22.16
Mq49252_c0_seq1	Q9LTA3	AT5G49690	UDP-glycosyltransferase superfamily protein	3.82	–
Mq49864_c0_seq8	Q8RXN0	...	WBCH protein 11	–	9.88
Mq41199_c0_seq1	Q8RXN0	AT1G17840	WBCH protein 11	–	8.68
Mq48967_c0_seq1	Q8GT20	AT1G78990	HXXXD-type acyl-transferase family protein	3.26	–
Mq48621_c0_seq1	Q70PR7	AT1G24430	HXXXD-type acyl-transferase family protein	–	4.57
Mq42937_c0_seq1	P43156	AT3G49340	Cysteine proteinases superfamily protein	5.69	–
Mq51367_c0_seq1	P52777	...	Phenylalanine ammonia-lyase	4.64	–
Pathogenesis-related (PR) protein					
Mq38660_c0_seq2	P09761	AT3G04717	Chitin-binding protein (PR-4)	–	10.35
Mq52623_c0_seq1	P81370	AT4G11650	Osmotin 34 (PR-5)	–	11.18
Mq44909_c0_seq3	E3SU11	AT4G11650	Osmotin 34 (PR-5)	–	7.95
Mq39168_c0_seq1	O23044	AT2G39040	Peroxidase (PR-9)	–	9.95
Mq35186_c0_seq1	Q9FX85	AT1G49570	Peroxidase (PR-9)	–	5.88
Mq33842_c0_seq1	P42813	AT1G14220	Ribonuclease (PR-10)	–	12.03

species such as those in the Myrtaceae family often exhibit high levels of nucleotide diversity in natural populations (Külheim et al. 2009). As a result, DE analysis packages might have discarded many candidate *A. psidii* resistance-related genes that were biologically significant in some individuals in our study, leading to a limited representation of the spectrum of defense mechanisms likely to be present in certain individuals of *M. quinquenervia*. Nonetheless, this study unveiled diverse pathways of defense in *M. quinquenervia*, which may be modulated in various ways by each genotype during pathogen invasion to produce an effective resistance. In our study, numerous defense-related genes determined by DE packages as significantly differentially expressed across all resistant individuals (compared with susceptible plants) were only greatly induced in two of the four individuals. After consideration of the DEG profile for each comparison, we propose that resistant individuals of *M. quinquenervia*

may have two different pathways to become resistant: (i) through specific recognition of the pathogen leading to induction of pathogen-related defenses and (ii) through preformed barriers, which could be either physical or chemical.

Some individuals of resistant *M. quinquenervia* may have molecular recognition pathways which then led to induced defenses to *A. psidii*. Based on the results from this study, it is feasible that resistant individuals 1 and 4 (HR1 and HR4) had primarily an induced defense to *A. psidii*. For example, a transcript (Mq47245_c0_seq3) encoding an LRR-RLK was only greatly induced in resistant individuals 1 and 4 (Fig. 8A; Table 4). In addition, resistant individuals 1 and 4 showed a more pronounced induction of a GST (Mq41846_c0_seq3) (Fig. 8D; Table 4), which suggested that the plants may have undergone oxidative burst, because GST detoxifies byproducts of lipid hydroperoxidases (Gechev et al. 2006; Levine

Fig. 8. Variation in expression of defense-related gene candidates among highly susceptible (HS) and highly resistant (HR) biologically independent samples (1 to 4); dpi = days postinoculation. **A**, Leucine-rich repeat receptor-like kinase; **B**, cysteine-rich kinase; **C**, nucleotide-binding site leucine-rich repeat protein; **D**, glutathione S-transferase; **E**, phenylalanine ammonia-lyase; **F**, flavanone 3-hydroxylase; **G**, WRKY transcriptional regulator; **H**, pathogenesis-related protein 1; and **I**, pathogenesis-related protein 5.

et al. 1994). Comparably, the transcriptome study of Soria-Guerra and colleagues (2010) observed an upregulation of GST in resistant *G. tomentella* upon soybean rust *Phakopsora pachyrhizi* infection. Resistant individuals 1 and 4 also had several transcripts which seemed to be markedly more highly expressed than the transcripts in the other two resistant plants at 5 dpi, yet were not detected by the DE analysis. These include transcripts encoding for PAL and F3H (Fig. 8). The PAL is involved in the committing biosynthetic step to the formation of phenylpropanoids, which includes a variety of antifungal compounds in the groups of stilbene, isoflavonoids, anthocyanins, flavanols, and condensed tannins (Dixon and Paiva 1995; Moore et al. 2014). The F3H, which is downstream of PAL in the phenylpropanoid pathway, functions in the branch that synthesizes flavanols and condensed tannins (Moore et al. 2014). We hypothesized that induced defense to *A. psidii* may have incorporated the phenylpropanoid pathway but, because we observed discrepancies in expression across resistant and susceptible samples for all copies of *PAL*, we could not conclude that the phenylpropanoid pathway was the determining factor of resistance against *A. psidii*. On the other hand, transcripts which encoded potential defense-enacting compounds such as PR genes of groups PR-1, PR-2, PR-3, PR-4, and PR-5 were more strongly induced and expressed in resistant individuals 1 and 4. This indicated diverse amplitudes of gene expression of PR proteins among biological independent resistant samples. Reorganizations of microtubules may also contribute to induced defense against *A. psidii*. Kobayashi and colleagues (1994) found that *Linum usitatissimum* L. (flax) susceptible to flax rust maintained the same microtubule arrangement as uninfected controls, whereas resistant flax showed evidence of microtubule reorganization. In our study, only resistant plants had the GO term ‘microtubule cytoskeleton organization’ enriched at 5 dpi (Fig. 6). Furthermore, after examining of all 31 transcripts that were assigned to this term, resistant individuals 1 and 4 had all but three transcripts (Mq45142_c1_seq24, Mq45142_c1_seq35, and Mq45142_c1_seq46) upregulated and more highly expressed than it was in resistant individuals 2 and 3 (HR2 and HR3).

Another mode of resistance to *A. psidii* in some individuals of *M. quinquenervia* might be preformed defenses through means of physical barriers or chemical constituents. Because resistant individuals 2 and 3 (HR2 and HR3) seemed to only have small changes in global gene expression after *A. psidii* infection (Fig. 2), we speculate that these plants may possess mainly preformed resistance against *A. psidii*. The histological study by Melander and Craigie (1927) showed that the thickness of cuticles and outer epidermal walls of *Berberis* spp. influenced its resistance to the stem rust (*Puccinia graminis*). Constitutive chemical compounds such as terpenes that may be toxic to pathogens are often another component in effective preformed defense (Wittstock and Gershenzon 2002). An example is peppermint (*Mentha × piperita*), which accumulates high amounts of terpenes in glandular trichomes that may be released upon disruption by pathogens (Wittstock and Gershenzon 2002). Further studies such as electron micrograph measurements of cuticle density and assessments of constitutive chemical constituents such as cuticular waxes may elucidate the basis of preformed barriers in *M. quinquenervia* against *A. psidii* (Serrano et al. 2014).

There are other instances of plants that did not coevolve with an invasive pathogen having an effective induced defense response, including North American white pine (*Pinus* spp.) infected by white pine blister rust (WPBR; *Cronartium ribicola*). Similar to our study, the transcriptome study by Liu and colleagues (2013) found that WPBR-resistant western white pine (*Pinus monticola*) overexpressed RLK, NBS-LRR proteins, transcriptional regulators, and PR proteins after rust infection. Hypotheses were proposed relating to the resistance of North American white pine to the WPBR, including ancient genes being retained or resistance genes being selected by the interaction with an endemic pathogen (Tobias et al. 2016). Vogan and Schoettle (2015) suggested that the resistance in

native plants such as white pine evolved for other biotic or abiotic stress that occurs in the plants’ native environment. This may suggest that resistance against *A. psidii* mediated by induced defense in *M. quinquenervia* could be related to another biotic stress such as endemic pathogenic or nonpathogenic fungi. Further work is needed to support such a hypothesis in *M. quinquenervia*. It is also worth noting that the expanding host range of *A. psidii* could be due to the phylogenetic relatedness between native host plants in South America and naïve plants in Australia and elsewhere (Longdon et al. 2014). There is a consensus that the host range of pathogens is dependent on the phylogenetic distance between the plants, which determines the pathogens’ ability to infect across species (Schulze-Lefert and Panstruga 2011).

This study highlights the molecular basis of defense in an ecologically important nonmodel woody plant species (*M. quinquenervia*) to an exotic pathogen (*A. psidii*). In this study, one of the aims was to find potential molecular markers for resistance selection. The aforementioned transcript (Mq47245_c0_seq3) encoding an LRR-RLK (Fig. 8A) may be a candidate marker, because it showed consistency in expression in all four resistant individuals before infection and low to no expression across susceptible plants, with the significance of the differences being confirmed by DE packages (Table 4). Further characterization of similar candidate genetic markers in this study would be of interest, because it would not only validate a defense role against *A. psidii* but also may be incorporated into plant breeding programs for reforestation purposes in the future. In conclusion, the diverse nature in expression of defense-related factors among resistant plants indicates the complexity of the defense mechanism in *M. quinquenervia*, and the sum of many factors contributed to the resistance of *M. quinquenervia* to *A. psidii*.

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