Dual RNA-Sequencing of *Eucalyptus nitens* during *Phytophthora cinnamomi* Challenge Reveals Pathogen and Host Factors Influencing Compatibility

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Damage caused by *Phytophthora cinnamomi* Rands remains an important concern on forest tree species. The pathogen causes root and collar rot, stem cankers, and dieback of various economically important *Eucalyptus* spp. In South Africa, susceptible cold tolerant *Eucalyptus* plantations have been affected by various *Phytophthora* spp. with *P. cinnamomi* considered one of the most virulent. The molecular basis of this compatible interaction is poorly understood. In this study, susceptible *Eucalyptus nitens* plants were stem inoculated with *P. cinnamomi* and tissue was harvested five days post inoculation. Dual RNA-sequencing, a technique which allows the concurrent detection of both pathogen and host transcripts during infection, was performed. Approximately 1% of the reads mapped to the draft genome of *P. cinnamomi* while 78% of the reads mapped to the *Eucalyptus grandis* genome. The highest expressed *P. cinnamomi* gene in planta was a putative crinkler effector (*CRN1*). Phylogenetic analysis indicated the high similarity of this *P. cinnamomi CRN1* to that of *Phytophthora infestans*. Some CRN effectors are known to target host nuclei to suppress defense. In the host, over 1400 genes were significantly differentially expressed in comparison to mock inoculated trees, including suites of pathogenesis related (PR) genes. In particular, a PR-9 peroxidase gene with a high similarity to a *Carica papaya* PR-9 ortholog previously shown to be suppressed upon infection by *Phytophthora palmivora* was down-regulated two-fold. This PR-9 gene may represent a cross-species effector target during *P. cinnamomi* infection. This study identified pathogenicity factors, potential manipulation targets, and attempted host defense mechanisms activated by *E. nitens* that contributed to the susceptible outcome of the interaction.

**Keywords:** plant defense, RNA-seq, *Eucalyptus nitens*, pathogenesis related genes, crinkler
INTRODUCTION

Species of the Oomycete genus *Phytophthora* are the most economically important pathogens of plants worldwide (Zentmyer, 1983; Erwin and Ribeiro, 1996). Of critical concern to the forestry industry is *Phytophthora cinnamomi* Rand, one of the most pathogenic and damaging species that affects agriculture, forestry, and native forests worldwide (Linde et al., 1994; Wingfield et al., 2011) and caused extensive damage to native ecosystems including *E. marginata* forests in Western Australia (Podger et al., 1967; Burgess et al., 1999) and native vegetation of the Western Cape of South Africa (Vonbroembsen and Kruger, 1985). Eucalypt plantations contribute significantly to the economy of South Africa (Godsmark, 2009). Susceptible cold-tolerant *Eucalyptus* plantations have been affected by various *Phytophthora* species to such an extent that some valuable species such as *E. fastigata* and *E. fraxinoides* are no longer cultivated (Linde et al., 1994; Wingfield and Kemp, 1994). *Eucalyptus nitens* is considered resistant to *P. cinnamomi* in its native environment but succumbs to the pathogen in plantations (Cahill et al., 2008). *P. cinnamomi*, an introduced stramenopile pathogen (Adl et al., 2005, 2012) severely affected stands of *E. nitens* in South Africa (Maseko, 2010).

Plant-pathogen interactions in forest systems have not been as well studied at a genomic level as it has for herbaceous crops. The recent availability of the genome sequence of *E. grandis* (Myburg et al., 2014) has provided a valuable resource for transcriptomic studies to dissect defense responses in related *Eucalyptus* species. RNA sequencing (RNA-seq) has contributed knowledge towards several host responses during pathogen challenge (Xu et al., 2011, 2012; Downen et al., 2012; Martinelli et al., 2012; Savory et al., 2012; Tremblay et al., 2012). Unlike previous probe based methods which require separation of host and pathogen cells, RNA-seq has allowed the study of both host and pathogen transcriptomics simultaneously. This technique, known as dual RNA-seq (reviewed in Westermann et al., 2012) allows the detection of minute amounts of pathogen RNA. It does not require predesigned species specific probes and is more sensitive than the previous methods of microarrays and northern blotting (Kunjeti et al., 2012; Tierney et al., 2012; Westermann et al., 2012; Camilios-Neto et al., 2014; Choi et al., 2014; Hayden et al., 2014). Pathogen RNA-seq data can be further mined for clues to pathogenicity (ability to cause disease) and virulence (degree of damage or pathology) factors based on functional genetics studies conducted in various host-pathogen interactions. The plant-host interactions database provides such data for comparative analysis (Winnenburg et al., 2006).

An enhanced understanding of the defense response of plants to *P. cinnamomi* will facilitate the production of resistant plants (Eshraghi et al., 2014a). Several RNA-seq host response studies to *Phytophthora* spp. have been undertaken to date (Kunjeti et al., 2012; Ali et al., 2014; Chen et al., 2014). The host responses of raspberry to *P. rubi*, and those of potato tubers to *P. infestans*, were successfully profiled using RNA-seq (Ward and Weber, 2012; Gao et al., 2013). This technique has also been applied to a native forest system, with the response of the oak *Notholithocarpus densiflorus* to *P. ramorum* elucidated (Kunjeti et al., 2012; Ward and Weber, 2012; Gao et al., 2013; Ali et al., 2014; Chen et al., 2014; Hayden et al., 2014).

The genome sequence of *P. cinnamomi var. cinnamomi* is currently available as a draft assembly of 77.97 Mb and a sequence read coverage depth of 69.6x (Reeve, 2012, unpublished; JGI Project identity: 1003775). This is an invaluable resource that provides insight into pathogenicity determinants in this species. This has been demonstrated for other *Phytophthora* species sequenced genomes (e.g., *P. infestans*, *P. sojae*, *P. ramorum*, reviewed in Jiang and Tyler, 2012).

*Phytophthora* species are able to manipulate their hosts to their own advantage. For example, *P. infestans* manipulates its host to suit its life-style by suppressing the hypersensitive response (HR) in potato during its biotrophic phase, then manipulating the induction of HR during the necrotrophic phase (Bos et al., 2010; Gilroy et al., 2011). This type of manipulation could be a trend in other *Phytophthora* interactions (Belja et al., 2009; Porter et al., 2009). Effectors excreted during this interaction are primarily a family of proteins expressed in all plant pathogenic oomycetes, and RxLRs which is confined to only *Phytophthora* species (Stam et al., 2013b; Chen et al., 2014).

Host defense responses are mediated through different mechanisms, and the timing and degree to which these are activated could determine the outcome of the interaction between a plant and pathogen (Tao et al., 2003). A factor driving compatible interactions has been revealed by several studies to involve a mass down-regulation of defense genes (Schlink et al., 2010). Pathways associated with these genes include the defense hormone salicylic acid (SA) which is associated with biotrophic defense responses, jasmonic acid (JA), and ethylene (ET) which are associated with necrotrophic defense responses, and abscisic acid (ABA) which is associated with abiotic stress as well as the host-pathogen defense (Bari and Jones, 2009). The host responses elicited by *P. cinnamomi* have been studied in different woody species at various levels including anatomical, physiological, biochemical, and molecular levels (recently reviewed in O’Jwual et al., 2014). Some of the major findings in these studies highlight the importance of correctly regulated HR and reactive oxygen species (ROS) and synthesis of phenylpropanoid pathway-related substances such as flavonoids, gibberellic acid (GA) and lignin. PR-1 and PR-5 feature in several interactions as possible resistance factors.

Multiple pathogenesis related (PR) gene classes are differentially regulated against *Phytophthora* and are thought to be important for successful defense (Moy et al., 2004; Schlink, 2009; Attard et al., 2010). The over-expression of specific PR genes have conferred tolerance against various *Phytophthora* spp. (Alexander et al., 1993; Fagoaga et al., 2001; Sarowar et al., 2009; Pushin et al., 2010; Acharya et al., 2013; He et al., 2013).

The *E. nitens*–*P. cinnamomi* interaction provided a novel system to study a compatible host-pathogen interaction using a dual RNA-sequencing approach. The aim of this study was to (i) conduct pathogenicity factors produced by *P. cinnamomi* and to determine the *E. nitens* defense response to the pathogen, and (ii)
to identify host genes that could potentially be suppressed by the pathogen to promote susceptibility. We observed, among other responses, high expression of a putative *P. cinnamomi* crinkler effector in planta and the down-regulation of a PR-9 gene, which may represent a common host effector target in *E. nitens*; two factors possibly contributing to the susceptible outcome of the interaction.

**METHODS**

**Inoculated Plant Material**

*E. nitens* seedlings were obtained from parents that were part of a third generation commercial breeding program (Sappi Forests Research, Shaw Research Centre, KwaZulu-Natal, South Africa). The criterion for selection was based on wood density gain. An MLRelate analysis, using the microsatellite markers developed by Faria et al. (2010), determined that the individuals from the Sappi breeding population showed relatively greater similarity to each other than those within the natural Australian *E. nitens* population (Melissa Reynolds, Forest Molecular Genetics, University of Pretoria). Previous sampling in *E. nitens* stands in South Africa have shown root rot due to *P. cinnamomi* (Maseko, 2010).

The *E. nitens* seedlings were grown in pine-bark seedling mix until stem thickness was >0.5 cm (~1 year), then stem inoculated with *P. cinnamomi* (CMW26310, Forestry and Agricultural Biotechnology Institute culture collection). A 4 mm cork borer and mycelial plug on V8 agar [CMV; modified from Erwin and Ribeiro (1996): 200 ml/L V8 juice (Campbell Soup Company, Camden, New Jersey), 20 g/L CaCO₃ (Merck), 20 g/L agar] was used for inoculations. Mock-inoculated plants were treated identically to infected trees, with a sterile V8 agar plug. Inoculation sites were covered with damp sterile cheesecloth, tinfoil, and Parafilm (Parafilm, Chicago, IL).

Stems were inoculated at two sites, 10 cm apart. In the attempt to represent the typical responses to *P. cinnamomi* in commercially grown *E. nitens*, for both the mock-inoculated control and inoculated trees, stem tissue was harvested from 18 trees (which consisted of three pooled biological replicates of six trees each) at 5 days post inoculation (dpi). Three centimeters of stem tissue was harvested per inoculation site, with 1.5 cm of stem tissue below and above the center of the site. Harvested material was immediately frozen in liquid nitrogen.

Since sampling was destructive, nine extra trees were used to observe symptom development for 6 weeks following inoculation. Beneath-bark lesions were measured in other trials to statistically validate the effectivity of inoculation. We observed, among other responses, high expression of a putative *P. cinnamomi* crinkler effector in planta and the down-regulation of a PR-9 gene, which may represent a common host effector target in *E. nitens*; two factors possibly contributing to the susceptible outcome of the interaction.

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putative orthologs were divided into up- and down-regulated datasets, which were analyzed for gene ontology (GO) over-representation in BINGO v2.4.4 (Maere et al., 2005) using the Cytoscape v2.8.3a platform (Shannon et al., 2003). Over-representation was evaluated against the Arabidopsis thaliana genome in the categories for “biological processes,” “molecular function,” and “cellular component.” A hypergeometric test with a Benjamini & Hochberg FDR correction of 0.05 was used. Understanding of biological pathways was aided by MapMan v3.5.1R2 (Thimm et al., 2004).

RT-qPCR
Genomic contamination in total RNA samples was removed by treating extracted total RNA samples with RNase-free DNaseI enzyme (Qiagen Inc., Valencia, CA). Total RNA samples were then purified using the RNeasy® MinElute Kit (Qiagen Inc.) and subsequently analyzed using a Bio-Rad Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA), to determine RNA integrity. The Improm-IITM Reverse Transcription System (Promega, Wisconsin, USA) was used to synthesize first strand cDNA from purified RNA samples. Primers were designed using Primer Designer 4 v4.20 (Sci Ed Central, Cary, North Carolina, USA). Primer pairs are indicated in Table 1.

Real-time quantitative reverse transcriptase PCR (RT-qPCR) was conducted according to the Minimum Information for Publication of RT-qPCR Experiments (MIQE) guidelines (Bustin et al., 2009) using a LightCycler® 480 Real-Time PCR system (Roche Diagnostics, GmBh, Basa, Switzerland) following parameters described in Naidoo et al. (2013). The qBASEplus v1.0 (Biogazelle NV, Belgium) software package was used to perform normalization and relative quantification. Significance was determined using a two-tailed Student’s t-test in Microsoft® Office Excel 2010.

Phylogenetic Analysis
Protein sequences were retrieved from GenBank, Phytozone and JGI, and aligned using MUSCLE (Edgar, 2004). RAxML (Stamatakis, 2006) was used to search for the best scoring maximum likelihood tree with rapid bootstrapping, with GAMMA BLOSUM62 as an evolutionary model. A Bayesian inference analysis was conducted in MrBayes 3.2.2 (Ronquist

Gene Ontology Over-Representation Analysis of Host Transcripts
Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to match significantly differentially expressed E. grandis v1.1 gene and transcript models to TAIR10 and TAIR9 identifiers based on a reciprocal BLAST analysis. The significantly differentially expressed genes (and transcripts for which the representative gene model was not differentially expressed) with TAIR10

| Eucalyptus ID* | Gene name | Forward primer | Reverse primer |
|---------------|-----------|----------------|---------------|
| Eu.0025320    | EgrWF71   | AAAGCAGGGAGGAGGTGGGATGAGAAGAA | TGCGAGCCCTGAGACCTGACAAGCT |
| Eu.020181     | EgrLR-RL7 | TGAGTAATCTGGGTACCTTGAAGC | GCCAGATTTGACGAGGCTCTGAACT |
| Eu.002533     | EgrNRT2.5 | TGTCGCAGAAGGACGACAAAGAAAGAA | GTAATCGTGCATCTGAACT |
| Eu.014195     | EgrPR-3   | GTATGTGGACTGCACTTGCATAGGG | CATTGGCCGTCAGTTATAG |
| Eu.017171     | EgrAF     | TGGTCTGACCTGAGTGGGCCAAGG | TAGAAGCAAGAGAGAATAG |
| Eu.020264     | EgrARA    | TGACGAGAGACTGACGAGG | GTTGCGACGGTCCTGAGATG |
| Eu.011100     | EgrMLO    | TGACGAGAGACTGACGAGG | GTTGCGACGGTCCTGAGATG |
| Eu.017171     | EgrAF     | TGGTCTGACCTGAGTGGGCCAAGG | CATTGGCCGTCAGTTATAG |
| Eu.020264     | EgrARA    | TGACGAGAGACTGACGAGG | GTTGCGACGGTCCTGAGATG |
| Eu.011100     | EgrMLO    | TGACGAGAGACTGACGAGG | GTTGCGACGGTCCTGAGATG |

*Eucalyptus grandis identities according to www.phytozome.net.
and Huelsenbeck, 2003). Two chains were sampled once every thousand generations out of a total of one million generations. Trees were summarized with a 10% burn-in. All programs used were housed in Geneious software package version 7.1.5.

RESULTS

*P. cinnamomi* infected tissue for RNA-seq profiling was obtained by inoculating the stems of *E. nitens* seedlings and harvesting at 5 dpi. At this time-point, slight browning was visible around inoculation sites of control plants, whereas lesions of inoculated plants extended to the boundaries of the 3 cm sampled area (Figure 1A). At 6 wpi lesion length was pronounced compared to the mock-inoculated controls (Figure 1B). At 4 wpi, ~50% of the inoculated plants showed mortality. Lesion length was measured for live plants only. The efficacy of inoculation was verified by the presence of hyphae in stem tissue and lesion development over a 4 day time-course (Figure 1C).

RNA-Sequencing and Mapping to *Phytophthora cinnamomi* and *Eucalyptus grandis* Genomes

Approximately 36 million reads were obtained per sample, and reads were mapped to the *E. grandis* genome version 1.1 (Table 2). In addition to host transcripts, we mapped *P. cinnamomi* transcripts expressed in planta, based on the draft *P. cinnamomi* assembly. A low percentage of mapping (0.08%) was observed in the control (mock inoculated) samples, which were considered conserved eukaryotic gene sequences. On average, 1% of the reads mapped to the *P. cinnamomi* genome in the inoculated samples. Approximately 78% of the transcripts derived from both inoculated and mock-inoculated *E. nitens* samples mapped to the *E. grandis* genome (Table 2). The number of expressed genes and the average FPKM values were similar across the three biological replicates of each treatment which provided confidence that the samples were treated in a consistent manner and the results were comparable across data sets (Table 2).

*Phytophthora cinnamomi* Genes Expressed in Planta

The list of transcripts expressed in the three inoculated *E. nitens* samples with CV < 0.2 are provided as a Supplementary File (Table S1). Of these 283 genes, the genes with hits (E-value ~0) to the plant host interactions database described as loss in pathogenicity, avirulence determinants, or reduced virulence are indicated in Table 3. Several of these have homology to transcripts with known roles in pathogenicity and virulence based on functional genetics experiments in other pathogen species. The highest expressed gene was a member of the CRN family protein. The *P. cinnamomi* putative CRN (JGI: Phyci261170) was aligned with other putative and described CRN family members and a maximum likelihood phylogenetic tree describing the relationship was produced. A congruent topology was recovered from Bayesian inference. The closest relationship was to a putative CRN protein from *P. ramorum*. Both the *P. ramorum* and *P. cinnamomi* putative CRN proteins are closely related to the better characterized *P. infestans* CRN proteins (Figure 2).

Differentially Expressed Genes in *Eucalyptus nitens*

After mapping to the genome, Cuffdiff was used to compare pathogen-inoculated *E. nitens* samples with the mock-inoculated controls. A total of 890 up-regulated and 585 down-regulated gene models were observed at a false discovery rate (FDR) of 0.05. The expression of a sub-set of differentially expressed genes *EgrWRKY75, EgrPR-3, EgrNRT2.5, EgrMLO, and EgrLRR-RLK7* were validated using RT-qPCR (Figure 3). The tissue for the RT-qPCR validation was sourced from a separate trial that was set up identically to the first trial, with three biological repeats. The expression patterns were comparable to RNA-seq results, with the correlation coefficient between the RNA-seq and the RT-qPCR expression being 0.73.

Over-Represented Gene Ontologies

Differentially expressed genes with matching TAIR10 IDs were used in BiNGO to test for over-representation against the *A. thaliana* genome as background. In the up-regulated dataset

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**FIGURE 1** | Symptom development in *Eucalyptus nitens* following challenge with *Phytophthora cinnamomi*. (A) A section of 1.5 cm stem tissue was harvested below and above the site of inoculation at 5 dpi. (i) Mock-inoculated and (ii) inoculated. (B) Lesions on *E. nitens* seedlings 6 wpi with *P. cinnamomi*. The small letters indicate that lesions on inoculated seedlings were significantly larger than the mock-inoculated negative control at p < 0.05 using the Mann–Whitney test for non-parametric data. Error bars show standard error based on n = 12 replicates. (C) Confocal microscopy of a longitudinal stem section showing *P. cinnamomi* hyphae (white arrows) at 4 dpi.
for biological processes, the majority of over-represented GO terms were related to defense (Figure 4). Several of the categories involved JA and ET signaling, and there were a few terms related to SA (Figure 4 and Table S1). Phenylpropanoid pathway terms and aromatic compound synthesis related to flavonoid biosynthesis were also found in this dataset (Figure 4 and Table S2). Another prominent term in the up-regulated dataset was response to water deprivation/water stress terms.

Over-represented GO terms in the down-regulated dataset for biological processes (Figure 5) were predominantly related to growth, cell wall modifications, and cell wall chemistry. The phenylpropanoid pathway terms were related to lignin biosynthesis, as opposed to flavonoid synthesis in the up-regulated dataset (Figure 5 and Table S2). There were minimal biotic stress-related terms, and hormone-related terms in this dataset such as auxin and gibberelin were apparent (Figure 5 and Table S1). Photosynthesis-related terms were also over-represented.

One of the noteworthy aspects of the RNA-seq data obtained was that several putative PR genes displayed particularly high fold-change values, mostly in the up-regulated dataset. This is summarized in Table 4. Within the E. nitens- Phytophthora cinnamomi interaction, the most prominent putative PR genes were PR-1, PR-3 (chitinase), and PR-5 (thraumatin-like and osmotin). These were not only consistently highly up-regulated in inoculated tissue, but there were multiple E. nitens putative orthologs per A. thaliana gene that are all regulated at similar fold-change levels. Putative E. nitens orthologs of PR-4 (chitin-binding), PR-8 (chitinase class III), and PR-12 (defensins) were up-regulated. The PR-9 (peroxidase) and PR-10 (ribonuclease-like) classes contained a mix of up- and down-regulated putative orthologs. PR-14 (lipid transfer proteins) and PR-15 (oxalate oxidase/germin) putative orthologs were all down-regulated.

The E. nitens putative PR-9 ortholog (Eucgr.E04056) was down regulated by a 1.85 fold-change on a log2 scale in the Phytophthora cinnamomi-inoculated tissue (indicated in bold in Table 4). The sequence of this ortholog was compared to peroxidase from Carica papaya (EL784270) and A. thaliana. The amino acid alignment is indicated in Figure 6. The E. nitens PR-9 sequence shared 80% amino acid identity with the C. papaya PR-9 sequence.

**DISCUSSION**

The compatible interaction between E. nitens and P. cinnamomi provided a model system to study the compatible interaction between Eucalyptus spp. and Phytophthora spp. One year old E. nitens plants were stem inoculated using P. cinnamomi and pronounced lesions were obtained (Figure 1) suggesting that successful infection had occurred. This was corroborated with the presence of hyphae in the stems as early as 48 hpi in some instances and subsequently consistently observed at 5 dpi. In order to gain a better understanding of this compatible pathosystem, a dual RNA sequencing approach, as described previously for other plant-pathogen interactions (Hayden et al., 2014), was undertaken to concurrently detect pathogenicity factors and host responses.

Analysis of transcripts mapping to the P. cinnamomi genome confirmed the presence of ~1% pathogen in the tissue profiled. We obtained 78% mapping of the E. nitens transcripts to the E. grandis genome which was similar to that obtained in a study by Ward and Weber (2012) where raspberry transcripts were mapped to the strawberry genome. This substantiates the use of cross-species resources in the event that no such genomic resources are available for the species of interest.

**Table 3** indicates possible determinants expressed in planta which may contribute to the susceptible outcome of the interaction. A pathogen transcript encoding a CRN family protein was highly expressed in planta. In Phytophthora species, the CRNs are a complex family of large proteins and various experiments suggest that some CRNs are able to target host factors to suppress plant defenses (Adhikari et al., 2013). The P. cinnamomi CRN is closely related to the CRN1 protein from P. infestans, suggesting that it may have the same role as described in P. infestans. Torto et al. (2003) showed that the P. infestans CRN1 and CRN2 effectors were expressed during infection of tomato and that CRN1 and CRN2 were able to cause necrosis in tobacco and CRN2 induced PR1a expression in tomato. While some CRN effectors are known to target host nuclei (Stam et al., 2013a) the role in virulence may be diverse in various life stages of P. infestans (Resjö et al., 2014). Liu et al. (2011) described two CRN effectors from P. sojae where one induced cell death and the other suppressed cell death in soybean. More recently, it was

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**Table 2** | Flagstat and FastQC RNA-seq mapping statistics of Eucalyptus nitens reads to the v1.1 Eucalyptus grandis and Phytophthora cinnamomi var cinnamomi genomes.

| Sample name | Total reads mapped to E. grandis (%) | Properly paired to E. grandis (%) | Singletons mapped to E. grandis (%) | % GC content | Expressed genes in host | Average FPKM in host | Total reads mapped to pathogen | % reads mapped to pathogen |
|-------------|-------------------------------------|---------------------------------|----------------------------------|-------------|------------------------|----------------------|-----------------------------|--------------------------|
| Control 1   | 37444809                            | 64.44                           | 10.24                            | 50          | 29024                  | 493598               | 30756                       | 0.08                     |
| Control 2   | 36111678                            | 68.02                           | 10.16                            | 50          | 29250                  | 492972               | 29274                       | 0.08                     |
| Control 3   | 37090251                            | 68.76                           | 8.90                             | 49          | 29135                  | 467006               | 29116                       | 0.08                     |
| Inoculated 1| 37234371                            | 66.13                           | 11.65                            | 49          | 29429                  | 471923               | 444935                      | 1.19                     |
| Inoculated 2| 36622434                            | 67.30                           | 12.48                            | 49          | 29407                  | 497117               | 552202                      | 1.51                     |
| Inoculated 3| 36022978                            | 68.19                           | 10.18                            | 49          | 29576                  | 473466               | 196534                      | 0.55                     |

aNumber of proper pairs in proportion to the total reads mapped.
bNumber reads where one from a pair in proportion to the total mapped.
| Gene identity | Average FPKM | Gene ontology | E-value | Gene name | Knock-out phenotype | PHI base accession |
|---------------|--------------|---------------|---------|-----------|---------------------|--------------------|
| e_gw1.822.3.1 | 1443.83      | gi|301096130[ref]XP_002897163.1|Cinkler (CRN) family protein | 3.85E-91 | cm1 | Effector plant avirulence determinant | 656 |
| eExt_fgenes1_pg.C_880034 | 1323.48 | hydrogen-transporting ATPase activity, rotational mechanism | 9.04E-26 | invC | Reduced virulence | 645 |
| eExtGenewise1.C_2370044 | 1096.91 | DNA binding | 5.16E-11 | GzLam002 | Reduced virulence | 1533 |
| e_gw1.40.157.1 | 950 | zinc ion binding, glutathione peroxidase activity, response to oxidative stress | 8.14E-35 | MoHYR1 | Reduced virulence | 2356 |
| fgenes1_kg.2__22_Locus05v1rpmk39.38 | 915.58 | protein binding, transcription factor binding, GTP binding, ATP binding | 1.40E-67 | CLPT1 | Reduced virulence | 339 |
| e_gw1.76.61.1 | 853.95 | catalytic activity, hydrolase activity, ATP binding, ATPase activity, ATPase activity | 7.85E-55 | PMR1 | Reduced virulence | 440 |
| eExtGenewise1.C_610064 | 741.59 | protein kinase activity, GTP binding, protein-tyrosine kinase activity | 1.27E-22 | MoSNF1 | Reduced virulence | 1058 |
| fgenes1_kg.16__102_Locus840v1rpmk311.24 | 731.62 | catalytic activity, FAD binding, oxidoreductase activity | 3.59E-04 | ALO1 | Reduced virulence | 197 |
| fgenes1_kg.9__117_Locus955v1rpmk35.55 | 647.81 | catalytic activity, cofactor binding, oxidoreductase activity | 4.47E-32 | MGG | Reduced virulence | 881 |
| fgenes1_pg.124__4 | 620.76 | FAD binding, oxidoreductase activity, cell redox homeostasis, electron transport | 7.82E-06 | SIDA | Reduced virulence | 1010 |
| gm1.2704_g | 421.24 | catalytic activity, ATP binding, metabolism | 0.01 | ACL2 | Loss of pathogenicity | 2387 |
| gm1.12073_g | 324.79 | nucleotide triphosphatase activity, nucleotide binding, hydrolase activity | 2.38E-66 | PEX6 | Loss of pathogenicity | 226 |
| gm1.756_g | 315.86 | catalytic activity, metabolism | 6.30E-17 | SICl | Reduced virulence | 2321 |
| gm1.272_g | 294.34 | catalytic activity, acetate-CoA ligase activity, AMP binding, etabolism | 3.33E-22 | AKT1 | Loss of pathogenicity | 133 |
| e_gw1.31.72.1 | 268.44 | microtubule motor activity, ATP binding, microtubule-based movement | 2.50E-32 | KIN2 | Reduced virulence | 465 |
| e_gw1.1.234.1 | 254.89 | catalytic activity, metabolism | 9.49E-12 | SICl | Reduced virulence | 2321 |
| e_gw1.93.22.1 | 254.29 | protein binding, protein kinase activity, protein-tyrosine kinase activity | 5.50E-22 | Ste11 | Loss of pathogenicity | 2484 |
| e_gw1.500.1 | 204.35 | antioxidant activity, oxidoreductase activity | 5.61E-04 | TSA1 | Reduced virulence | 386 |
| eExtGenemark1.C_2810025 | 203.63 | electron-transfer-flavoprotein dehydrogenase activity, electron transport | 0 | SIDA | Loss of pathogenicity | 486 |
| e_gw1.67.108.1 | 199.31 | ATP binding | 1.08E-57 | LHS1 | Reduced virulence | 2058 |
| e_gw1.82.257.1 | 198.02 | ATP binding | 3.65E-13 | LHS1 | Reduced virulence | 2058 |
| e_gw1.108.166.1 | 195.13 | protein kinase activity, protein-tyrosine kinase activity | 2.83E-66 | McCMK1 | Reduced virulence | 2158 |
| e_gw1.2.24.1 | 187.23 | helicase activity, nucleic acid binding, ATP dependent helicase activity, ATP binding | 9.73E-70 | VAD1 | Reduced virulence | 423 |
| e_gw1.184.44.1 | 185.29 | protein kinase activity, protein-tyrosine kinase activity, protein serine/threonine kinase activity | 1.67E-51 | SNF1 | Reduced virulence | 198 |
| fgenes1_pg.66__13 | 184.79 | catalytic activity, aspartic-type endopeptidase activity, metabolism | 1.15E-31 | SICl | Reduced virulence | 2321 |
| MIX7251_264_83 | 180.35 | polygalacturonase activity, carbohydrate metabolism | 0 | Pcpig2 | Reduced virulence | 2343 |
| gm1.8948_g | 164.67 | ATP binding, nucleotide binding, nucleoside triphosphatase activity, tRNA ligase activity | 2.70E-47 | ABC4 | Loss of pathogenicity | 2067 |
| e_gw1.2.738.1 | 153.76 | hydrolase activity, cellulose binding, serine-type endopeptidase activity, blood coagulation | 1.21E-46 | CBEL | Effector plant avirulence determinant | 660 |
| fgenes1_pg.112__14 | 142.08 | gi|325187184[emb]CCA21725.1|bromodomain containing 2 putative [Albugula laibachii Nc14] | 1.63E-09 | GzBrom002 | Reduced virulence | 1317 |
| e_gw1.11.45.1 | 137.55 | phosphotransferase activity, alcohol group as acceptor | 1.02E-31 | VPS34 | Loss of pathogenicity | 195 |
| e_gw1.74.48.1 | 116.37 | protein binding, transcription factor binding, GTP binding, ATP binding, GTPase activity | 5.62E-40 | CLPT1 | Reduced virulence | 339 |
| eExt_fgenes1_pg.C_1470005 | 110.97 | transporter activity, binding, ATPase activity, ATP binding, transport | 1.15E-26 | MgAtr4 | Reduced virulence | 310 |
| gm1.14921_g | 103.14 | motor activity, ATP binding, myosin | 3.16E-114 | GzWing020 | Reduced virulence | 1648 |
shown that the two effectors together, or one alone, can suppress host defenses by interacting with catalases and in so doing, modulate the HR and H$_2$O$_2$ levels in planta (Zhang et al., 2015). Another well characterized example of an apoplastic effector is that of the Cellulose Binding, Elicitor, and Lectin-like (CBEL) transcript, which elicits necrosis and defense gene expression in hosts and is necessary for attachment onto plant surfaces (Mateos et al., 1997; Séjalon-Delmas et al., 1997). Further examination of the virulence factors and the host targets they affect, will provide insight into the cause of the host responses observed in E. nitens.

The over-represented GO terms in E. nitens challenged with P. cinnamomi (Figures 4, 5) were indicative of a host actively attempting to combat infection, albeit at a late stage of a compatible interaction. We observed differential expression of various defense responses, but highlight two possible factors contributing to susceptibility.

Some physiological responses to P. cinnamomi include the down-regulation of photosynthesis-related terms and up-regulation of water stress terms. Various tree species inoculated with P. cinnamomi show declines in stomatal conductance and photosynthesis. In Eucalyptus sieberi, this decline is associated with susceptibility, since these factors decreased less severely in resistant Eucalyptus sideroxylon (Dempsey et al., 2012). In Quercus suber, photosynthesis and stomatal conductance also decreased after inoculation (Medeira et al., 2012). Manter et al. (2007) noted the prevalence of photosynthetic and stomatal conductance decreases in Phytophthora-host interactions. They showed that photosynthetic decline could be caused by elicitors in the absence of water stress. Maintaining adequate photosynthetic levels may assist tolerance or resistance, which is a possible explanation of why lower photosynthetic rates and stomatal conductance is associated with an increase in pathogen (Portz et al., 2007) and lower tolerance to Phytophthora spp. (Reeksting et al., 2014).

Distinct components of the phenylpropanoid pathway are present in both the up- and down-regulated datasets. There are several up-regulated genes with GO annotations associated with flavonoid biosynthesis. Susceptible Lupinus angustifolius up-regulated the flavonoid genistin in response to P. cinnamoni (Gunning et al., 2013) however, certain Citrus flavonoids have an antimicrobial action against Phytophthora citrophthora (del Río et al., 2004). In the down-regulated dataset, GO terms associated with lignin synthesis via the phenylpropanoid pathway are over-represented. For lignin synthesis, most genes encoding enzymes involved in biosynthesis of the coniferyl alcohol (G subunit) and sinapyl alcohol (S subunit) are down-regulated, although genes encoding enzymes catalyzing the synthesis of coumaryl alcohol (H subunit) are up-regulated (Table S2). Since synthesis of the S and G subunits is possibly suppressed, lignin biosynthesis could be down-regulated in E. nitens. Lignin is associated with strengthening of cell-walls and helps prevent penetration by a pathogen (Bechinger et al., 1999), and down-regulation of monolignols can compromise host resistance (Naoumkina et al., 2010). Lignin synthesis plays a role in raspberry responses to challenge with P. rubi (Ward and Weber, 2012) and transgenic potato plants with limited phenylpropanoid substrates had increased susceptibility to P. infestans (Yao et al., 1995).

Gene ontology terms pertaining to JA, SA, and ET pathways were over-represented in the up-regulated dataset in E. nitens, suggesting that they could play a role in defense signaling for this interaction. Other studies involving hosts inoculated with Phytophthora spp. have also shown mixed hormone responses (Attard et al., 2010; Shibata et al., 2010). JA may be needed for successful defense against P. cinnamomi in maize, a resistant monocot (Allardyce et al., 2013). Terms related to GA and auxin were over-represented in the down-regulated dataset. Treatment of soybean with GA increased susceptibility to P.
sojae (Sugano et al., 2013) and it has been proposed that GA influences defense against necrotrophic fungi by repressing resistance (Mengiste, 2012). The role of auxin in defense against P. cinnamomi has recently been described (Eshraghi et al., 2014b), where Arabidopsis auxin sensitivity and transport mutants were shown to be highly susceptible to the pathogen. Treatment of L. angustifolius with an inhibitor of auxin transport increased susceptibility to P. cinnamomi.
Since PR genes are markers of defense hormone signaling, the different putative PR genes expressed reflect the mix of JA and SA signaling noted in the over-representation analysis.

Gene models identified as PR genes in this dataset are putative orthologs of A. thaliana PR genes. For many of these genes, there are multiple differentially expressed E. grandis gene models matching to one A. thaliana putative ortholog and an expansion of several PR genes in E. grandis has been noted (Naidoo et al., 2014). Expression of these multiple PR gene transcripts in E. nitens could indicate that some of the orthologs have slightly different functions and are all used during a defense response.

Transcription of PR-1, chitinase (PR-3), chitin-binding protein (PR-4), and thaumatin-like protein/osmotin (PR-5) putative orthologs appears to be highly up-regulated in E. nitens (Table 4). A Phytophthora-resistant potato expresses PR-1 constitutively (Ali et al., 2012) and constitutive expression of PR-1 in transgenic tobacco confers resistance to P. parasitica (Alexander et al., 1993). Transgenic plants over-expressing PR-5 genes have been shown to increase resistance to P. citrophthora and P. infestans (Fagoaga et al., 2001; Pushin et al., 2010; Acharya et al., 2013).

Peroxidases (PR-9) are potential cross-species Phytophthora effector targets, since a certain C. papaya peroxidase (EL784270) and its putative orthologs have been suppressed in different hosts upon inoculation with P. sojae, P. palmivora, and P. infestans (Moy et al., 2004; Restrepo et al., 2005; Porter et al., 2009). An E. grandis gene, Eucgr.E04056, is highly similar to the C. papaya ortholog and is also strongly suppressed in the current interaction. Putative PR-14 and PR-15 orthologs were down-regulated in E. nitens and while these orthologs would...
TABLE 4 | Expression of pathogenesis related genes in response to *Phytophthora cinnamomi* inoculation in *Eucalyptus nitens*.

| Gene family   | TAIR ID   | E. grandis ID | Description                                             | log2 expression |
|---------------|-----------|---------------|---------------------------------------------------------|-----------------|
| **PR-1**      | AT2G14580.1 | Eucgr.D01152  | Basic pathogenesis-related protein 1                   | 4.36            |
|               | AT2G14580.1 | Eucgr.G01140  | Basic pathogenesis-related protein 1                   | 4.40            |
|               | AT2G14580.1 | Eucgr.G01148  | Basic pathogenesis-related protein 1                   | 4.37            |
|               | AT2G14580.1 | Eucgr.D01160  | Basic pathogenesis-related protein 1                   | 4.36            |
|               | AT2G14580.1 | Eucgr.G01171  | Pathogenesis-related gene 1                            | 4.31            |
|               | AT2G14610.1 | Eucgr.G01134  | Basic pathogenesis-related protein 1                   | 3.34            |
|               | AT2G14610.1 | Eucgr.G01137  | Pathogenesis-related gene 1                            | 3.24            |
|               | AT2G14610.1 | Eucgr.L02505  | Pathogenesis-related gene 1                            | 3.14            |
|               | AT2G14610.1 | Eucgr.L01707  | Pathogenesis-related gene 1                            | 3.13            |
| **PR-3**—Chitinase class I, II, IV, VI, VII | AT3G12500.1 | Eucgr.L00941  | Basic chitinase                                          | 4.02            |
|               | AT3G12500.1 | Eucgr.J02519  | Basic chitinase                                          | 3.94            |
|               | AT3G12500.1 | Eucgr.L00938  | Basic chitinase                                          | 3.93            |
|               | AT3G54420.1 | Eucgr.H00326  | Homolog of carrot EP3-3 chitinase                       | 3.75            |
|               | AT3G54420.1 | Eucgr.H00321  | Homolog of carrot EP3-3 chitinase                       | 3.75            |
|               | AT3G12500.1 | Eucgr.L00939  | Basic chitinase                                          | 3.73            |
|               | AT3G54420.1 | Eucgr.H00328  | Homolog of carrot EP3-3 chitinase                       | 3.57            |
|               | AT3G12500.1 | Eucgr.L00937  | Basic chitinase                                          | 3.47            |
|               | AT3G12500.1 | Eucgr.L01495  | Basic chitinase                                          | 3.11            |
|               | AT3G54420.1 | Eucgr.K02166  | Homolog of carrot EP3-3 chitinase                       | 2.53            |
|               | AT3G54420.1 | Eucgr.K02166  | Homolog of carrot EP3-3 chitinase                       | 2.21            |
|               | AT3G54420.1 | Eucgr.A00020  | Homolog of carrot EP3-3 chitinase                       | 1.43            |
|               | AT1G05850.1 | Eucgr.H00455  | Chitinase family protein (TAIR 9)                       | −0.75           |
|               | AT3G16920.1 | Eucgr.H04034  | Chitinase-like protein 2                                | −1.33           |
| **PR-4**—Chitin-binding | AT3G04720.1 | Eucgr.B02124  | Pathogenesis-related 4                                 | 3.42            |
|               | AT3G04720.1 | Eucgr.L03258  | Pathogenesis-related 4                                 | 3.32            |
|               | AT3G04720.1 | Eucgr.B02122  | Pathogenesis-related 4                                 | 3.06            |
| **PR-5**—Thaumatin-like and osmotin | AT1G20030.2 | Eucgr.E01382  | Pathogenesis-related thaumatin superfam                 | 5.42            |
|               | AT1G20030.2 | Eucgr.E01384  | Pathogenesis-related thaumatin superfam                 | 5.31            |
|               | AT1G20030.2 | Eucgr.E01389  | Pathogenesis-related thaumatin superfam                 | 5.23            |
|               | AT1G20030.2 | Eucgr.E01385  | Pathogenesis-related thaumatin superfam                 | 5.13            |
|               | AT4G11650.1 | Eucgr.H03863  | Osmotin 34                                              | 5.09            |
|               | AT1G20030.2 | Eucgr.E01381  | Pathogenesis-related thaumatin superfam                 | 4.96            |
|               | AT4G11650.1 | Eucgr.H03865  | Osmotin 34                                              | 4.93            |
|               | AT4G11650.1 | Eucgr.H03864  | Osmotin 34                                              | 4.77            |
|               | AT4G11650.1 | Eucgr.L01962  | Osmotin 34                                              | 4.69            |
|               | AT4G11650.1 | Eucgr.E00567  | Osmotin 34                                              | 4.43            |
|               | AT4G11650.1 | Eucgr.D01888  | Osmotin 34                                              | 3.57            |
|               | AT4G11650.1 | Eucgr.D01892  | Osmotin 34                                              | 3.34            |
|               | AT4G11650.8 | Eucgr.D01887  | Osmotin 34                                              | 3.31            |
|               | AT4G11650.9 | Eucgr.E00560  | Osmotin 34                                              | 2.97            |
|               | AT5G38280.1 | Eucgr.A01474  | PR5-like receptor kinase                                | 1.03            |
|               | AT5G38280.1 | Eucgr.A01470  | PR5-like receptor kinase                                | 0.96            |
|               | AT5G38280.1 | Eucgr.A01478  | PR5-like receptor kinase                                | 0.78            |
|               | AT2G29790.1 | Eucgr.J02061  | Pathogenesis-related thaumatin superfam                 | −1.18           |
|               | AT4G38660.1 | Eucgr.G01772  | Pathogenesis-related thaumatin superfam                 | −1.33           |
|               | AT1G3620.1  | Eucgr.B00944  | Pathogenesis-related thaumatin superfam                 | −1.36           |
| **PR-8**—Chitinase class III | AT5G24090.1 | Eucgr.E00091  | Chitinase A                                             | 2.40            |
|               | AT5G24090.1 | Eucgr.L03478  | Chitinase A                                             | 1.52            |

(Continued)
up and down regulated genes are indicated as a gradient from bright red to bright green.

have to be characterized further, it is tempting to speculate that their suppression may be driving susceptibility. For example, enhanced resistance to \textit{P. nicotianae} was conferred by a pepper lipid transfer protein over-expressed in tobacco (Sarowar et al., 2009) and \textit{PR-15} may encode for a germin-like oxalate oxidase known to produce hydrogen peroxide that is toxic to pathogens (van Loon et al., 2006; Ferreira et al., 2007).

**CONCLUSION**

The outcomes of this dual RNA-seq study provided valuable insights into \textit{P. cinnamomi} pathogenicity and virulence factors and \textit{E. nitens} defense mechanisms utilized against \textit{P. cinnamomi}. Several factors may contribute to the compatibility however, we have used further sequence and functional genetics support to motivate that the \textit{P. cinnamomi CRN} and the \textit{E. nitens PR-9} genes are important contributors to the susceptible outcome. Future work involving comparison with a resistant interaction over a time-course is required to provide an indication of host targets manipulated by \textit{P. cinnamomi} and to enhance understanding of the defense pathways required for resistance.

**AUTHOR CONTRIBUTIONS**

FM, LS, SiN, and TM performed the experimental work, conducted and interpreted data analyses. SaN conceived the study, obtained funding to support the research. AM provided input into the experimental design and technical aspects of RNA-sequencing and assisted with critical evaluation of the manuscript. FM, LS, and SaN wrote the manuscript with input from DB and NV, who supervised aspects of this research.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00191
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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