Dual RNA-Seq Analysis of the Pine-Fusarium circinatum Interaction in Resistant (Pinus tecunumanii) and Susceptible (Pinus patula) Hosts

Erik A. Visser 1, Jill L. Wegrzyn 2, Emma T. Steenkamp 1, Alexander A. Myburg 1,* and Sanushka Naidoo 1,*

1 Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), Centre for Bioinformatics and Computational Biology, University of Pretoria, Private Bag X20, Pretoria 0028, South Africa
2 Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA
Correspondence: sanushka.naidoo@fabi.up.ac.za

Received: 3 July 2019; Accepted: 20 August 2019; Published: 4 September 2019

Abstract: Fusarium circinatum poses a serious threat to many pine species in both commercial and natural pine forests. Knowledge regarding the molecular basis of pine-F. circinatum host-pathogen interactions could assist efforts to produce more resistant planting stock. This study aimed to identify molecular responses underlying resistance against F. circinatum. A dual RNA-seq approach was used to investigate host and pathogen expression in F. circinatum challenged Pinus tecunumanii (resistant) and Pinus patula (susceptible), at three- and seven-days post inoculation. RNA-seq reads were mapped to combined host-pathogen references for both pine species to identify differentially expressed genes (DEGs). F. circinatum genes expressed during infection showed decreased ergosterol biosynthesis in P. tecunumanii relative to P. patula. For P. tecunumanii, enriched gene ontologies and DEGs indicated roles for auxin-, ethylene-, jasmonate- and salicylate-mediated phytohormone signalling. Correspondingly, key phytohormone signaling components were down-regulated in P. patula. Key F. circinatum ergosterol biosynthesis genes were expressed at lower levels during infection of the resistant relative to the susceptible host. This study further suggests that coordination of phytohormone signaling is required for F. circinatum resistance in P. tecunumanii, while a comparatively delayed response and impaired phytohormone signaling contributes to susceptibility in P. patula.

Keywords: Dual RNA-seq; ergosterol biosynthesis; Fusarium circinatum; host-pathogen interaction; Pinus patula; Pinus tecunumanii

1. Introduction

One of the most important pathogens to natural and industrial pine forests is the pitch canker fungus, Fusarium circinatum Nirenberg and O’Donnell [1–3]. Since the first report of the pathogen, and the disease it causes, in the southeastern US, F. circinatum has been identified in more than ten countries world-wide, resulting in significant losses in both nurseries and plantations [2–4]. The pathogen was originally identified due to the visible symptom development on pines and classified as a necrotrophic pathogen of pine, capable of infecting douglas fir (Pseudostuga menziesii) and up to 60 different species of pine, including economically important species such as Pinus pinaster, Pinus taeda and Pinus radiata [3]. Alarmingly, the last natural stands of P. radiata are under threat of extirpation due to pitch canker [5]. Multiple studies, however, have since shown a broader range of potential ecological activities [6]. The pathogen has been shown to endophytically infect pine [7], as well as certain grass species [8], maize [9] and some dicots [10]. Thus, there is a high likelihood for F. circinatum to spread into unaffected regions through naturally occurring inoculum reservoirs on unmonitored species [2,7–9].
Sterols are important lipids in eukaryotic cellular membranes with vital roles in regulating membrane fluidity and permeability. Ergosterol is present in the membranes of many fungi and is required for fungal growth [11,12]. This sterol has also been shown to play a crucial role in vegetative differentiation and virulence in Fusarium graminearum [13]. Plant PR-1 proteins have been shown to affect pathogen growth through binding and sequestration of sterols. While effective against sterol-auxotrophic pathogens such as oomycetes, sterol-prototrophic pathogens such as fungi only become sensitive to PR-1 when their sterol biosynthesis is compromised [14].

The most extensively planted softwood in South African forestry is Pinus patula, which is highly susceptible to F. circinatum [15]. As a result, cultivation of this species declined by ca. 14% between 2002 and 2016 [16,17] due to high post-planting mortality rates [18,19]. Current long-term control strategies under investigation include the usage of alternative species, breeding and selection programs to produce more resistant families and hybridisation between susceptible and resistant species [15,19]. Commercial deployment of hybrids between P. patula and Pinus tecunumanii from low elevation provenances, which are resistant to F. circinatum, has already started [15,20,21]. Knowledge of the molecular mechanisms underlying host resistance and susceptibility, as well as pathogen virulence, could expedite development of resistant genotypes and improve the effectiveness of genetic resistance.

Ongoing advancements in high-throughput sequencing (HTS) technology and bioinformatics has allowed a more in-depth investigation of transcriptomic responses in non-model plants such as pine. A transcriptome wide analysis in Pinus monticola implicated calcium and abscisic acid signaling, as well as down-regulation of photosystems and carbon fixation in resistance to the biotrophic rust fungus Cronartium ribicola [22]. A recent transcriptome wide study on F. circinatum-challenged P. radiata identified increased expression of genes associated with abscisic acid, salicylic acid and ethylene response pathways, in resistant relative to susceptible seedlings [23]. The increasing sensitivity of HTS technologies has also made it feasible to simultaneously sequence expressed genes from both the plant and pathogen in a single sample, an approach referred to as dual RNA-seq, thus allowing parallel investigation of host and pathogen responses during an interaction [24–27].

In a previous study, transcriptomes were assembled for P. tecunumanii and P. patula during F. circinatum challenge, however, host and pathogen gene expression was not investigated [28]. This study aimed to elucidate molecular mechanisms underlying host resistance by examining pathogen and host responses during F. circinatum challenges in pine.

2. Results

2.1. Annotation

Mercator annotation assigned MapMan functional categories to 34,502 of 52,735 (65%) P. patula transcripts and 18,906 of 28,621 (66%) P. tecunumanii transcripts. Coding regions were predicted for 14,423 F. circinatum transcripts using GeneMarkS-T and best-hit selection of BLASTp hits resulted in alignments for 12,985 (90%) proteins (Table S1). The majority of best hits (12,558) originated from Fusarium species. EggNOG annotation assigned 13,948 (97%) F. circinatum sequences to families and InterProScan annotation assigned domains to 9405 (65%) proteins, resulting in a total of 14,185 (98%) annotated sequences, of which 5368 (37%) were assigned gene ontology (GO) terms. Only 4516 (31%) F. circinatum sequences had putative alignments to PHI-base (Table S2). GhostKOALA assigned KO numbers to 27,973 (53%) P. patula transcripts, 12,038 (42%) P. tecunumanii transcripts and 4225 (29%) F. circinatum transcripts (Table S3).

2.2. Transcriptome Profiling

An average of 69.9 ± 6.8% P. patula reads mapped to the Pipt_v2.0 transcriptome and 71.3 ± 7.8% P. tecunumanii reads mapped to the Pnte_v1.0 transcriptome (Table S4). Additionally, for 3- and 7-dpi respectively, an average of 0.04 ± 0.01% and 0.05 ± 0.03% reads from mock-inoculated P. patula samples mapped to the F. circinatum reference transcriptome, with 0.08 ± 0.01% and 0.82 ± 0.42% reads mapped
from inoculated samples. Comparably for P. tecunumanii, an average of 0.02 ± 0.01% and 0.30 ± 0.17% reads mapped to the F. circinatum transcriptome from mock-inoculated samples, with 0.16 ± 0.04% and 1.62 ± 0.81% reads mapped from inoculated samples.

Filtering of the expression data identified 25,000, 20,614 and 5,003 expressed genes for P. patula, P. tecunumanii and F. circinatum, respectively, across all samples. Subsequent filtering of F. circinatum expression resulted in 4,354 high-confidence expressed genes (Table 1, Table S5). Differential expression (DE) analysis of inoculated samples (P. tecunumanii versus P. patula) identified 132 and 470 significant F. circinatum differentially expressed genes (DEGs) for 3- and 7-dpi, respectively (Table 1, Table S5). P. patula DE analysis identified 323 and 7453 significant DEGs (inoculated versus mock-inoculated) at 3- and 7-dpi, while 735 and 2499 significant DEGs were identified for P. tecunumanii (Table 1, Tables S6 and S7).

Table 1. Summary of significant differentially expressed genes identified for each comparison.

| Category | Genes Up-Regulated a | Genes Down-Regulated a |
|----------|----------------------|------------------------|
| Differentially expressed host genes b | | |
| P. patula 3-dpi | 209 | 114 |
| P. patula 7-dpi | 4116 | 3337 |
| P. tecunumanii 3-dpi | 625 | 110 |
| P. tecunumanii 7-dpi | 1987 | 512 |
| F. circinatum high confidence expressed genes c | | |
| 3-dpi P. patula samples | 210 | 0 |
| 7-dpi P. patula samples | 2372 | 5 |
| 3-dpi P. tecunumanii samples | 1409 | 0 |
| 7-dpi P. tecunumanii samples | 4125 | 1 |
| Differentially expressed F. circinatum genes d | | |
| 3-dpi inoculated samples | 39 | 93 |
| 7-dpi inoculated samples | 264 | 206 |

a Significant (FDR < 0.05), up- (log2(Fold Change) > 0.5) and down-regulated (log2(Fold Change) < −0.5), differentially expressed genes identified using the Wald test (Benjamini & Hochberg FDR correction) with DESeq2.

b Host genes differentially expressed in inoculated relative to mock-inoculated host expression data.

c F. circinatum genes differentially expressed in inoculated relative to mock-inoculated samples in the full expression data set (including both host and pathogen mapped reads) for each host. Up-regulated genes represent high confidence F. circinatum expressed genes. Down-regulated genes were excluded from downstream analysis.

d F. circinatum genes differentially expressed in P. tecunumanii relative to P. patula inoculated samples from pathogen expression data.

2.3. Over-Represented GO Terms within Pathogen Datasets

GO enrichment analysis in the high confidence expressed F. circinatum genes showed shared biological process (BP), cellular compartment (CC) and molecular function (MF) terms between all data sets related to ribosomes, translation and lipid metabolism, indicative of growth, as well as BP terms related to responses to farnesol (Figure S3, Table S8). BP terms related to pectin hydrolysis were only enriched in the 3-dpi P. patula data set. Most enriched terms in the P. tecunumanii 3-dpi data set were also enriched in both the P. tecunumanii and P. patula 7-dpi data sets. This included CC terms related to membranes and mitochondria, MF terms related to hydrolysis, lipid binding and oxidoreductases, and BP terms related to responses to oxidative stress and respiration (Figure S3, Table S8). For both hosts at 7-dpi, there were also many enriched BP terms related to localization, cell wall organization, alcohol biosynthesis and sterol biosynthesis.

Further GO enrichment analysis of F. circinatum DEGs between P. tecunumanii and P. patula at both timepoints was performed to identify differences in pathogen responses between hosts (Table S9). Few terms were enriched for F. circinatum genes up-regulated during infection of P. tecunumanii relative to P. patula. No terms were enriched in the 3-dpi data set, while there was enrichment for the CC term, extracellular region, and the BP terms, carbohydrate metabolic process, and, oxidation-reduction process, in the 7-dpi data set. In contrast, many terms were enriched for F. circinatum
genes down-regulated in inoculated P. tecunumanii relative to P. patula samples. At both time points, cytoplasmic translation terms were enriched in all three GO categories (Table S9). At 3-dpi, BP terms related to glycolysis and energy production were enriched, while at 7-dpi MF and BP terms related to sterol and alcohol biosynthesis were enriched. Due to the enrichment of terms related to ergosterol biosynthesis in the high confidence 7-dpi data set from both hosts, as well as in the down-regulated 7-dpi data set, this pathway was investigated further.

2.4. Transcriptional Responses Related to Ergosterol Biosynthesis in the Pathogen

Candidate genes for all ergosterol biosynthesis steps could be identified in the F. circinatum transcriptome except HMG-CoA synthase (Table S10). Due to the physiological importance of HMG-CoA synthase, this likely indicates incompleteness of the genome rather than absence of the gene. ERG10 and ERG20 showed significantly lower expression during infection of P. patula than P. tecunumanii at 7-dpi (Figure 1). While this indicates that F. circinatum produces more farnesyl diphotphate (FDP) during infection of P. tecunumanii, FDP is the precursor for a wide array of metabolites. Conversely, five genes involved in late ergosterol biosynthesis, CYP51/sterol-14α-demethylase (ERG11), C-4 methylsterol oxidase (ERG25), sterol 24-C-methyltransferase (ERG6), C-8 sterol isomerase (ERG2) and δ7-sterol 5-desaturase (ERG3), were expressed at lower levels during infection of P. tecunumanii than P. patula at 7-dpi (Figure 1).

2.5. Over-Represented GO Terms within Host Datasets

Few GO terms were enriched for P. patula DEGs at 3-dpi (Table S11). Analysis of up-regulated DEGs identified the CC terms, cell wall and external encapsulating structure, as well as the BP terms, syncytium formation, cytoplasmic translation, response to oxygen-containing compound and response to chitin. All DEGs underlying the enriched syncytium formation term were predicted expansins, proteins involved in cell wall relaxation. The only enriched GO term in the down-regulated DEGs at 3-dpi was the MF term xyloglucan:xyloglucosyl transferase activity, an enzymatic activity involved in cell wall reinforcement.

For 7-dpi up-regulated DEGs, the enriched CC terms shared between timepoints were mostly related to the cytoskeleton, coated vesicles, vacuoles and the proteasome (Table S12). Most enriched MF terms shared between timepoints, as well as the terms unique to 3-dpi, were related to protein and nucleic acid binding (Table S12). The MF terms specific to the 7-dpi up-regulated DEGs were predominantly related to hydrolases, including chitinase activity, and lipases. The 7-dpi unique MF terms contained the term, xyloglucan:xyloglucosyl transferase activity, enriched in the 3-dpi down-regulated P. patula dataset.
Figure 1. Expression of *Fusarium circinatum* ergosterol biosynthesis genes during infection. The y-axes represent average FPKM across biological replicates for inoculated samples from *P. patula* (blue) and *P. tecunumanii* (green) at 3- (dark colours) and 7- (light colours) dpi. Error bars represent the standard
error of the mean (n = 3). Letters above bars indicate significant difference in expression (FDR < 0.05). Dashed black outlines indicate high-confidence expressed genes. ERG10 = acetyl-CoA acetyltransferase, HMG1 = 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, ERG12 = mevalonate kinase, ERG8 = phosphomevalonate kinase, MVD1 = mevalonate pyrophosphate decarboxylase, IDI1 = isopentenyl diphosphate:dimethylallyl diphosphate isomerase, ERG20 = geranylgeranyl diphosphate synthase, ERG9 = farnesyl-diphosphate farnesyltransferase, ERG1 = squalene epoxidase, ERG7 = lanosterol synthase, ERG11 = CYP51/sterol-14α-demethylase, ERG24 = 614-sterol reductase, ERG25 = C-4 methylsterol oxidase, ERG26 = sterol-4-α-carboxylate 3-hydrogenase, ERG27 = 3-keto sterol reductase, ERG6 = sterol 24-C-methyltransferase, ERG2 = C-8 sterol isomerase, ERG3 = δ7-sterol 5-desaturase, ERG5 = CYP61a/sterol 22-desaturase, ERG4 = δ24(24(1))-sterol reductase. ERG13 (HMG-CoA synthase) was absent from the transcriptome.

A large number of BP terms were enriched in the P. tecunumanii up-regulated DEGs at both time points (Table S12). Potential defence related BP terms enriched at both timepoints were related to response to chitin, ubiquitin-mediated proteolysis (UMP) and vesicle mediated transport. Enriched terms unique to the 3-dpi dataset were related to UMP, ET signaling, JA mediated ISR and terpenoid biosynthesis. The enriched terms unique to 7-dpi were related to the SA response, SA-mediated SAR, JA responses, ET biosynthesis and the biosynthesis of various phytoalexins, including camalexin.

Enriched GO terms for down-regulated P. tecunumanii DEGs at both timepoints were mainly related to cellular growth and replication (Table S12). Most enriched BP terms for down-regulated DEGs at 3-dpi were also enriched for down-regulated DEGs at 7-dpi.

2.6. Transcriptional Responses Related to Host Phytohormone Signalling

Phytohormones play crucial roles during growth and development. Interactions between these hormones also serve to regulate gene expression during stress responses. Hormone biosynthesis and signaling related DEGs were investigated to identify putative pathways involved in the pine-Fusarium interaction due to the enrichment of different phytohormone related GO terms in the up-regulated data at 3- and 7-dpi in P. tecunumanii, as well as in both the up- and down-regulated data at 7-dpi in P. patula (Figure 2, Tables S13 and S14).

2.6.1. Cytokinin

At both time points in P. tecunumanii, there was up-regulation of UDP-glycosyl transferase (UGT) and cytokinin oxidase/dehydrogenase (CKX) genes, enzymes related to cytokinin (CK) degradation, with more UGTs up-regulated at 7-dpi (Figure 2, Table S14). There was also down-regulation of cytochrome P450s (CYP) related to CK biosynthesis and histidine kinase (HK) receptor genes at 7-dpi. Increased degradation and down-regulation of biosynthesis indicate suppression of CK at both time points. In P. patula, the only CK related DEGs at 3-dpi were up-regulated UGT genes, while at 7-dpi, similar to P. tecunumanii, there was up-regulation of UGT and CKX genes as well as down-regulation of HK genes (Figure 2, Table S13). Furthermore, two A-ARR genes, negative regulators of CK signaling, showed up-regulation and two B-ARR genes, positive regulators of CK signaling and A-ARRs, showed down-regulation in P. patula at 7-dpi. This, together with the up-regulation of superoxide dismutase (SOD) genes, indicate suppression of CK signaling.

2.6.2. Gibberellic Acid

At 3-dpi in P. patula, the only gibberellic acid (GA) related DEG was an up-regulated GA methyl transferase (GAMT, Figure 2, Table S13). Unlike other phytohormones, methylation results in GA degradation (Eckardt, 2007). GA 3-oxidase (GA3ox) and putative ent-copalyl diphosphate synthase (CPS) genes were up-regulated at both time points in P. tecunumanii, as well as at 7-dpi in P. patula indicating possible GA biosynthesis at 7-dpi in both hosts. However, there were also down-regulated CPS genes, with more CPS genes down-regulated in P. patula than P. tecunumanii. GID1, GA2ox, LBD and AMY genes were up-regulated in both hosts at 7-dpi. A LFY gene was up-regulated at 7-dpi in
P. patula and phytochrome-interacting factor 3 (PIF3) genes were down. While up-regulation of LFY and AMY genes indicate the presence of GA signaling between 3 and 7-dpi, up-regulation of DELLA responsive GA2ox, GID1 and LBD indicate suppression of GA signaling at 7-dpi in both hosts.

Figure 2. Summary of phytohormone related host DEGs during F. circinatum challenge. Up- and down-regulation (inoculated relative to mock-inoculated) of genes related to cytokinin, abscisic acid, gibberellic acid, brassinosteroid, ethylene, jasmonic acid, salicylic acid and auxin signalling, in P. patula (blue) and P. tecunumanii (green) at three (dark colours) and seven (light colours) days post inoculation,
are indicated by arrows (↑ and ↓ respectively; Additional file 5: Tables S4 and S5). Dotted red lines indicate suppression, dashed black lines indicate positive interaction, solid black lines indicate enzymatic reactions. Borderless text indicates processes, square bordered text indicates proteins, round bordered text indicates compounds. AAO3—abscisic-aldehyde oxidase; A-ARR—type A Arabidopsis response regulator; ABA—abscisic acid; ABF—ABA response factor; ABI—ABA insensitive; ACAA1—acyl-Coenzyme A acyltransferase 1; ACC—1-aminocyclopropane-1-carboxylic acid; ACO—ACC oxidase; ACS—ACC 1-aminocyclopropane-1-carboxylic acid synthase; AHP—Arabidopsis histidine phosphotransferase; Ala—alanine; AMY—α-amylases; AOC—allene oxide cyclase; AOS—allene oxide synthase; ARF—auxin response factor; Asp—aspartic acid; Aux/IAA—auxin inhibitor; AUX/LAX—auxin influx carriers; BAK1—BR1-associated kinase; B-ARR—type B Arabidopsis response regulator; BIN2—BR insensitive 2; BR—brassinosteroid; BRH1—brassinosteroid-responsive RING H2; BRI1—brassinosteroid insensitive 1; BSK—BR-signalling kinase; BSU1—protein phosphatase BRII suppressor; CAS1—cycoartenol synthase; CDG1—constitutive differential growth 1; CHI—chalcone-flavone isomerase; CHS—chalcone synthase; CIPK—Cbl-interacting protein kinase; CK—cytokinin; CKX—CK dehydrogenase; COI1—coronatine insensitive 1; CYP—Cytochrome P450 family protein; DOGT1—DON-glucosyltransferase 1; EDS1—enhanced disease susceptibility 1; EIN—ET insensitive; ERF—ET response factor; ERG6—sterol C-methyltransferase; ET—ethylene; ETR1—ET receptor; GA—gibberellic acid; GA2ox—GA 2-oxidase; GA3ox—GA 3-oxidase; GAMT—GA methyl transferase; GH3—Gretchen Hagen3 family protein; GiD1—GA insensitive dwarf 1; Glu—glutamine; GST—glutathione-S-transferase; HK—histidine kinase; IAA—indole-3-acetic acid; IAMT—IAA methyl transferase; ILL—JA-ile/IAA amino acid hydrolase; IPT2—isopentenyl transferase; JAR1—JA-amino acid synthetase; JAZ—jasmonate zim-domain family transcription repressor proteins; JMT—JA methyl transferase; LBD—lob domain-containing protein; Leu—leucine; LFY—LEAFY; LOX—lipoxigenase; MeJAs—methyl-jasmomate; MES—methyl esterase; MeSA—methyl-salicylate; MFP2—multifunctional protein 2; MYC—JA responsive transcriptome factor; NCED—9-cis-epoxycarotenoid dioxygenase; NINJA—novel interactor of JAZ; NPR—non-expressor of PR; NSY—neoxanthin synthase; PIF3—phytochrome-interacting factor 3; PIN—auxin efflux transporter; PP2C—type 2C protein phosphatases; PR—pathogenesis related proteins; PYR/PYL—ABA receptors; RTE1—reversion-to-ethylene sensitivity 1; SA—salicylic acid; SAM—S-adenosyl-methionine synthetase; SAMT—SA methyl transferase; SAUR—small auxin up RNA protein; SCF—Skp-cullin-F-box complex; SDR—xanthoxin dehydrogenase; SMT2—24-methylenesterol C-methyltransferase; SOD—superoxide dismutase; TCH4—Xyloglucan endotransglycosylase hydrolase protein; TGA—TGA family transcription factors; TPL—topless; Ub—ubiquitin; UGT—UDP-Glycosyl/Glucoyl/Glucuronosyl transferase; UMP—ubiquitin mediated proteolysis; ZEP—zeaxanthin epoxidase.

2.6.3. brassinosteroids

A steroid biosynthesis gene was down-regulated at 7-dpi in *P. tecunumanii*, while brassinosteroid (BR) signalling kinase 1 (*BSK1*) was up-regulated (Figure 2, Table S14). At both time points in *P. tecunumanii*, there was also up-regulation of putative brassinosteroid-responsive RING H2 (*BRH1*) genes. In *P. patula*, some steroid biosynthesis and brassinazole resistant (*BZR*) genes, as well as *BRH1*, were up-regulated at 7-dpi; cycloartenol synthase (*CAST1*), brassinosteroid-insensitive 1 (*BRI1*), *BIM1* (a BZR2 synergist) and brassinosteroid-insensitive 2 (*BIN2*) genes, however, were down. BR signaling has been shown to rapidly decrease the expression of *BRH1*, while the pathogen elicitor chitin increases expression [29]. Thus, BR signaling appeared to be absent at 3-dpi in both hosts and up-regulation of *BRH1* at 7-dpi was indicative of BR signaling suppression. DON-glucosyltransferase 1 (*DOGT1*) genes, associated with inactivation of BRs and CKs through glucosylation [30,31], were up-regulated at 7-dpi in both species, further indicating suppression of both CK and BR signaling.
2.6.4. Abscisic Acid

In *P. tecunumanii* a 9-cis-epoxycarotenoid dioxygenase (NCED) was down-regulated at both time points, however, at 7-dpi another NCED, as well as a putative xanthoxin dehydrogenase (SDR) was up-regulated and an abscisic acid (ABA) hydroxylase was down-regulated (Figure 2, Table S14). This could indicate suppression of ABA biosynthesis at 3-dpi and induction at 7-dpi in the resistant host. In *P. patula*, an NCED and an ABA hydroxylase were up-regulated at 3-dpi. At 7-dpi, although an SDR1 was up-regulated, other ABA biosynthesis genes, zeaxanthin epoxidase (ZEP) and abscisic-aldehyde oxidase (AAO3), were down-regulated while an ABA hydroxylase and a carotenoid cleavage dioxygenase 8 (CCD8) were up-regulated. CCD8 diverts carotenoid metabolism away from Zeaxanthin. This indicates suppression of ABA biosynthesis and increased degradation at 7-dpi in the susceptible host.

ABA receptors were up-regulated at both time points in *P. tecunumanii* and at 7-dpi in *P. patula*. A type 2C protein phosphatase (PP2C) gene was down-regulated at 7-dpi in *P. patula*. The up-regulation of receptors and a CIPK20 could indicate ABA signaling at both time points in *P. tecunumanii*. At 7-dpi in *P. patula*, down-regulation of ABA biosynthesis indicates suppression of ABA levels, while up-regulation of receptor and CIPK20 genes with down-regulation of a PP2C suggest ABA signaling.

Cross talk between CK and ABA signaling is mediated by ABA-insensitive 4 (ABI4) and ABI5, positive regulators of ABA signaling, and A-ARRs. ABI5 activity is attenuated by interaction with A-ARRs, allowing A-ARRs to negatively regulate both CK and ABA signaling [32]. ABI4 positively regulates A-ARR5, resulting in suppression of CK responses by ABA signaling [33]. An ABI4 gene was down-regulated at 7-dpi in *P. tecunumanii*, which could indicate a lack of ABA signaling despite the up-regulation of receptors. As ABA has been implicated in suppression of GA responses by stabilizing DELLA proteins, preventing their degradation [34], the up-regulation of ABA biosynthesis in *P. tecunumanii* could be related to GA suppression.

2.6.5. Ethylene

1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), ACC oxidase (ACO) and a large amount of ET response factor (ERF) genes were up-regulated at both time points in *P. tecunumanii* and at 7-dpi in *P. patula* (Figure 2, Table S13). More ERFs were up-regulated at 3-dpi in *P. tecunumanii* than at 7-dpi. There was also a S-adenosyl-methionine (SAM) synthetase up- and an ET insensitive 2 (EIN2) gene down-regulated at 7-dpi in *P. patula*. The up-regulation of ET biosynthesis genes and ERFs indicates active ET signaling at both timepoints in *P. tecunumanii* and at 7-dpi in *P. patula*. However, down-regulation of EIN2 and up-regulation of a predicted reversion-to-ethylene sensitivity 1 (RTE1), a known negative regulator of ET signaling [35], in *P. patula* could interfere with ET signaling. ET signaling has been implicated in the suppression of ABA biosynthesis as well as negative regulation of ABA signaling [36]. Thus, despite up-regulation of ABA receptors at both time points in *P. tecunumanii*, up-regulation of ET biosynthesis could indicate suppression of ABA signaling by ET.

2.6.6. Jasmonic Acid

At 3-dpi in *P. patula*, a single jasmonate zim-domain (JAZ) family transcription repressor was down-regulated (Figure 2, Table S13). At 7-dpi in both species lipoxygenase 5 (LOX5), allene oxide synthase (AOS), allene oxide cyclase 3 (AOC3), 12-oxophytodienoate reductase 3 (OPR3), JA-Ile/IAA-amino acid hydrolases (ILL) and chalcone synthase (CHS) genes were up-regulated. Additionally, LOX1 and OPR2 were up-regulated in *P. patula*. JA-Ile 12-hydroxylases (CYP94B), JAZ and jasmonate methyl transferase (JMT) genes were up-regulated at both time points in *P. tecunumanii* and at 7-dpi in *P. patula*. Thus, few JA related genes were DE at 3-dpi in both species while at 7-dpi there was increased JA biosynthesis and responses. Although this indicates a role for JA at 7-dpi in both species, there was down-regulation of a coronatine insensitive 1 (COI1) gene at 7-dpi in *P. patula*, which could suppress JA responses.
2.6.7. Salicylic Acid

There are two main routes for SA production in plants, the isochorismate (IC) and phenylalanine ammonia-lyase (PAL) pathways, both originating from the shikimate pathway product chorismate. In *P. tecunumanii*, shikimate pathway and PAL genes were up-regulated at both time points, with more genes up-regulated at 7-dpi (Figure 2, Table S14). Phytoalexin deficient 4 (*PAD4*) was also up-regulated at both time points, however, enhanced disease susceptibility 1 (*EDS1*) was only up-regulated at 7-dpi. Conversely, in *P. patula*, while shikimate pathway and PAL genes were also up-regulated at 7-dpi, *EDS1* and the majority of *PAD4* DEGs were down-regulated. Isochorismate synthase (*ICS*), the first step in the IC pathway [37], was also down-regulated at 7-dpi. Furthermore, isochorismatase, an enzyme that diverts isochorismate away from SA synthesis, was up-regulated. PR-1 genes, classic SA response genes [38], were up-regulated at both time points in *P. tecunumanii*, while in *P. patula*, they were up-regulated at 7-dpi and down-regulated at 3-dpi (Tables S13 and S14). Thus, while SA signaling appeared to be active at 7-dpi in *P. tecunumanii*, at 7-dpi in *P. patula* there were indications of SA biosynthesis and signaling suppression.

2.6.8. Auxin

In *P. patula*, the only auxin related DEGs at 3-dpi were an up-regulated auxin response factor (*ARF*) 19 and a down-regulated small auxin up-regulated (*SAUR*)-like gene (Figure 2, Table S13). At 7dpi, an auxin biosynthesis gene (*YUCCA*) was down-regulated in *P. patula*. As *YUCCA* is only involved in one auxin biosynthesis pathway, auxin could still be produced via other routes [39]. *ARF4*, *ARF6*, *ILL5* (IAA-amino acid hydrolase), *CAND1* (Cullin-associated and neddylation dissociated 1) and three *Aux*/IAA auxin repressor genes were also down-regulated at 7-dpi in *P. patula*, indicating suppression of auxin signaling, despite the up-regulation of putative *SAUR* genes as well as *GH3* (Grethchen Hagen 3 acyl acid amido synthetase family proteins), *IAMT1* (IAA carboxyl methyltransferase 1) and *ILL6* genes. In *P. tecunumanii*, no auxin biosynthesis genes were differentially expressed, however, auxin influx carrier genes were up-regulated at both time points and an auxin efflux carrier gene was up-regulated at 3-dpi. Furthermore, *Aux*/IAA, *GH3* and *SAUR* genes were up-regulated at both time points and there was up-regulation of *IAMT*, *ILL6* and *CAND1* genes as well as down-regulation of some *Aux*/IAA and *SAUR* genes at 7-dpi. The up-regulation of *ILL6* and influx proteins indicate an increase of auxin levels in *P. tecunumanii* at both time points, however, the efflux carrier indicates lower auxin levels at 3- than 7-dpi, which is reflected in the amount of auxin response DEGs.

A *MES1* gene, a methyl esterase capable of hydrolyzing MeSA, MeJA and MeIAA, was up-regulated in both hosts at 7-dpi. There was also up-regulation of MES17, a MeIAA specific methyl esterase, at 7-dpi in *P. tecunumanii*. Systemic signaling through hormone methyl esters requires demethylation to activate the hormone [40–42]. Thus, up-regulation of *IAMT1* and *MES* genes could indicate systemic signaling at 7-dpi in both hosts.

At 7-dpi, cullin genes were up-regulated in *P. tecunumanii* but down-regulated in *P. patula*. Cullins are critical structural proteins of Skp-Cullin-F-box (SCF) complexes. This could indicate a decreased ability to activate defence signaling in *P. patula* as SCF complex catalyzed ubiquitination is an important component of GA, JA and auxin signaling [43–45].

3. Discussion

The disparate *F. circinatum* resistance phenotypes of *P. patula* and *P. tecunumanii* [15] provided a pathosystem to study resistant and susceptible host-pathogen interactions between *Pinus* spp. and *F. circinatum*. The susceptibility of *P. patula* and resistance of *P. tecunumanii* to *F. circinatum* challenge was previously confirmed by the significant difference in lesion development between species, as well as mortality of *P. patula* seedlings, while *P. tecunumanii* seedlings showed signs of recovery [28]. Significantly higher read mapping to the *F. circinatum* transcriptome from inoculated relative to mock-inoculated samples, and up-regulation of most genes in the *F. circinatum* high confidence
expressed genes, for each sample set supported the presence of pathogen sequence reads, as expected from the reported infection [28]. This was corroborated by higher mapping from 7-dpi relative to 3-dpi inoculated samples, which is indicative of fungal growth.

Expression of *F. circinatum* genes indicated compromised ergosterol biosynthesis during infection of the resistant host at 7-dpi, which could increase pathogen susceptibility to PR-1 proteins [14]. All detected *F. circinatum* orthologs of five ergosterol biosynthesis genes, ERG11, ERG25, ERG6, ERG2 and ERG3, were expressed at lower levels during infection of *P. tecunumanii* relative to *P. patula*. Azole group fungicides inhibit fungal growth by inhibiting ERG11, blocking ergosterol biosynthesis [46]. One known mechanism of azole resistance in *Candida albicans* results from a loss of function mutation of ERG3, without affecting virulence [47]. Conversely, in *F. graminearum* ERG3 mutation has been associated with decreased virulence [48]. Although many fungal pathogens exhibit resistance to azole fungicides, plant coumarins have also been associated with ERG11 inhibition in *C. albicans* [49], and the transformation of *Arabidopsis* and barley with an ERGII targeting double-stranded RNA, to elicit host-induced gene silencing, resulted in complete immunity to *F. graminearum* [50]. Additionally, a tomato glycoalkaloid has been associated with suppression of ergosterol biosynthesis in *Saccharomyces cerevisiae* by inhibiting ERG6 [51] and treatment of *C. albicans* with the terpenoid farnesol resulted in down-regulation of ERG11, ERG25, ERG6 and ERG3 [52]. Thus, the lower relative expression of these genes during infection of the resistant host could aid in host resistance.

Enriched GO terms indicated a delayed and imprecise response to *F. circinatum* challenge by *P. patula*. The overrepresented GO terms for *P. tecunumanii* DEGs suggested active host defence responses at both time points, while defence related GO terms were only enriched at 7-dpi for *P. patula*. Additionally, in *P. tecunumanii* there was enrichment of ubiquitin-mediated proteolysis and cell cycle regulation terms in the up-regulated DEGs and DNA and cellular replication terms in the down-regulated data which were absent for *P. patula*. Ubiquitin-mediated proteolysis is a critical process for the activation and regulation of GA, JA, SA and auxin signaling pathways [43–45,53]. Several lines of evidence suggest the existence of a growth-defence trade-off in plants [54], thus decreasing replication could assist the host in mounting a successful defence. Furthermore, although ET, JA and SA related terms were enriched for the up-regulated 7-dpi *P. patula* DEGs, similar to *P. tecunumanii*, there was also enrichment for terms related to oxidative stress, apoptosis and ROS production. In plants, these responses are associated with HR [55], which has been linked to increased susceptibility against necrotrophic pathogens and has been shown to be promoted by necrotrophs to facilitate infection [56].

Phytohormone related DEGs at 3-dpi indicated roles for auxin and ET in *P. tecunumanii* defence responses. Plant defence against necrotrophic pathogens is usually associated with active signaling by both the ET and JA pathways [57–60]. However, while no JA biosynthesis genes were up-regulated, putative JAZ genes (repressors of JA responses) and a JA hydroxylase (involved in JA degradation) were up-regulated, indicating suppression of JA signaling. The jasmonate-insensitive I (JIN1, a.k.a. AtMYC2) MYC protein has been shown to negatively regulate EIN3 expression, inhibiting the expression of ERF1 [61]. Thus, JA suppression could allow for the activation of a larger repertoire of ET responses. Auxin has also been shown to have an antagonistic effect on JA signaling by stimulating the expression of JAZ proteins [62].

At 7-dpi, *P. tecunumanii* DEGs suggested the inclusion of JA and SA in host resistance. JA biosynthesis genes were up-regulated, indicative of JA signaling and, while more ET biosynthesis genes were up-regulated, markedly fewer ERFs were up-regulated, reflecting the expected JA/ET antagonism [61]. Additionally, a larger array of auxin response genes were differentially regulated relative to 3-dpi. A synergistic interaction between auxin and JA signaling has been associated with enhanced host resistance to necrotrophic pathogens [45,63]. There was also up-regulation of SA biosynthesis genes and the SA response marker genes PAD4 and EDS1 [64,65], as well as more PR-1 genes at 7- relative to 3-dpi. Therefore, while at 3-dpi *P. tecunumanii* seemed to induce ET and auxin while suppressing JA signaling, at 7-dpi, transcriptomic responses indicated a complex regulation of
host defence using auxin, ET, JA and SA. Although SA signaling is usually classified as antagonistic to both auxin and JA signaling, synergistic interactions also exist [32,45,66]. SA signaling has been shown to induce JA biosynthesis and modulate JA defences in Arabidopsis effector-triggered immunity [67,68]. The early auxin responsive GH3 proteins play an important role in mediating crosstalk between auxin, JA and SA [45]. The core JA signaling component JAR1, which is required for production of the bioactive JA-Ile conjugate, is a GH3 [69], and increased GH3 expression has been shown to simultaneously induce the SA pathway and derepress the auxin pathway [70]. The SA conjugate salicyloyl-aspartate has also been implicated as a signaling molecule to induce systemic resistance [70,71]. Thus, auxin signaling could play an important role in coordinating and integrating phytohormone defence pathways, similar to the central role played in growth and development [45,72–74].

Host responses at 3-dpi in P. patula indicated a lack of defence and increased membrane permeability. Less than 400 genes were differentially expressed at 3-dpi in the susceptible host. A previous study investigating P. patula responses to F. circinatum challenge at 1-dpi identified even fewer DEGs [75]. Although overrepresentation of the GO term, response to chitin, at 3-dpi indicated fungal perception [76,77], there was no enrichment for defence related terms and few phytohormone related or PR family DEGs. Furthermore, there was overrepresentation of the GO term, syncytium formation, in the up-regulated DEGs and the term, xyloglucan:xyloglucosyl transferase activity, in the down-regulated DEGs. Expansins are involved in loosening of the cell wall associated with growth as well as symbiotic interactions [78,79]. Suppression of these proteins has been associated with increased resistance to necrotrophic pathogens [79,80]. Xyloglucan:xyloglucosyl transferases are involved in covalent cross-linking of cell wall polymers, such as xylose, and have been associated with cell wall reinforcement through xyloglucan remodeling [81,82]. Increased levels of cell-wall bound xylose have been associated with resistance to necrotrophic pathogens, while decreased levels have been associated with susceptibility, in Arabidopsis [83]. Thus, the up-regulation of expansins and the down-regulation of xyloglucan:xyloglucosyl transferases could contribute to susceptibility in P. patula by increasing membrane permeability. Combined with the seeming lack of defence responses, this could indicate effector triggered susceptibility at 3-dpi.

Despite the enrichment of defence related GO terms, P. patula DEGs at 7-dpi suggested an impaired phytohormone defence response against F. circinatum. Regarding SA related DEGs, the biosynthesis gene ICS2, as well as the SA response marker genes Edwardsia and PAD4 were down-regulated. Thus, unlike P. tecunumanii, the SA defence pathway appeared to be suppressed at 7-dpi. Biosynthesis genes for ET and JA, as well as many ERF and JAZ genes, were up-regulated. Auxin responsive SAUR and GH3 genes were up-regulated, while Aux/IAA and ARF genes were down-regulated. Although this could indicate signaling by these phytohormones, key JA and ET signaling genes, COI1, TPL and EIN2, were down-regulated and a negative regulator of ET signaling, RTE1, was up-regulated, indicating that these signaling pathways could be compromised. Auxin signaling has been shown to have an antagonistic effect on JA signaling by stimulating the expression of JAZ proteins [62,84,85] as well as inducing the expression of ERFs [86–88]. However, similar to JA and GA signaling, auxin signaling requires SCF-mediated repressor degradation [44,89]. Down-regulation of CAND1 and cullin genes suggests a decrease in available SCF complexes. Thus, despite the difference in the number of DEGs between time points, indicating a delayed response, immune signaling appeared to be compromised at 7-dpi. Nonetheless, the large number of up-regulated defence-related and PR-genes suggest some form of defence signaling. One of the down-regulated auxin response factors, ARF2, has been implicated in the negative regulation of COI1-independent defence responses in A. thaliana [90]. Similarly, the up-regulation of a rice GH3 has been associated with SA- and JA-independent immunity in rice [80]. Consequently, the differential regulation of defence related genes at 7-dpi could be the result of phytohormone-independent signaling.

In summary, this dual RNA-seq study suggested that ergosterol could be required for F. circinatum virulence in pine and identified phytohormone signaling pathways potentially involved in host resistance and susceptibility. This study purports that the observed reduction of F. circinatum ergosterol
biosynthetic gene expression could compromise this pathway, combined with the up-regulation of host PR-1 genes, this could be a key factor contributing to host resistance. Future work to determine the effect of suppressing F. circinatum ergosterol biosynthesis on host susceptibility and pathogen response to host metabolite treatment is required to provide further support for the observed responses. Furthermore, DEGs in P. tecunumanii indicated the integration and coordination of auxin, ET, JA and SA mediated defence responses could be required for resistance, while the absence of defence responses at 3-dpi and the down-regulation of phytohormone signaling components at 7-dpi in P. patula suggested pathogen inhibition of host responses. To the authors’ knowledge, these results represent the first comprehensive investigation of F. circinatum gene expression during pathogenesis of pine, as well as the first comparison of host responses to F. circinatum between two different pine species. Although an ideal comparison would have been to compare responses between resistant and susceptible genotypes of each species, no susceptible P. tecunumanii LE genotypes are known and even the most tolerant P. patula genotypes are still susceptible. Despite this limitation, the current approach improves knowledge on the pine-F. circinatum host pathogen interaction, as well as adding to the limited knowledge of defence responses in conifers. While this study investigated how these resistant and susceptible hosts respond to F. circinatum challenge on a transcriptomic level, under the assumption that changes in expression are implicated in defence, it is possible that differences in basal defences between the species would contribute to the resistant or susceptible outcome. Future work is required to investigate the role of physiological differences between these hosts.

4. Materials and Methods

4.1. Read Data From F. circinatum Inoculation Trial

RNA-sequencing libraries for P. patula and P. tecunumanii, generated from a F. circinatum inoculation trial, were obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA). In brief, six-month-old seedlings, from open-pollinated families, were inoculated with F. circinatum isolate FCC3579 and mock-inoculated using sterile glycerol [28]. The top 1 cm of shoot tissue was harvested at 3- and 7-days post inoculation (dpi) for three biological replicates per treatment group. A biological replicate consisted of tissue pooled from 16 seedlings. The complete disease progression was reported previously [28]. At 3- and 7-dpi, disease symptoms were not observed on either host species, however, at 14-dpi P. patula showed marked lesions from the point of inoculation. The expected difference in host resistance, represented by the difference in lesion development rates [15,28], was clearly visible by 21-dpi, with pronounced lesion development on inoculated P. patula and only mild discoloration on inoculated P. tecunumanii (Figure S1). RNA was extracted using the Plant/Fungi RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) and sent to Novogene (Novogene Corporation Inc, Chula Vista, CA, USA) for strand specific RNA-sequencing on an Illumina HiSeq2500 (Illumina, San Diego, CA, USA), PE125 for 3-dpi samples and PE150 for 7-dpi samples.

4.2. Reference Sequences

Host reference transcriptomes, Pipt_v2.0 for P. patula and Pnte_v1.0 for P. tecunumanii, were obtained from the NCBI Transcriptome Shotgun Assembly (TSA) database. Predicted proteins were assigned to MapMan functional categories using Mercator [91] with default parameters and inclusion of all available databases. The F. circinatum reference transcriptome was obtained by extracting the longest transcript sequence for all predicted genes (15,049) from the F. circinatum (strain FSP34) genome sequence [92]. The extracted transcript sequences were annotated with EnTAP debug_0.7.4.6 [93]. Open reading frame prediction was performed using GeneMarkS-T v5.1 March 2014 [94] followed by BLASTp similarity searches using the NCBI’s non-redundant protein database (release-84), RefSeq complete protein database (release-84) and the UniProtKB/Swissprot database (release-2017_09) through diamond 0.9.9 [95] (minimum-query-coverage = 80%, minimum target coverage = 60%, minimum e-value = 1 × 10^{-5}), as well as EggNOG 0.99.1 [96] and InterProScan 5.25-64.0 [97] for orthologous group and GO
term assignment (Figure S2). Predicted \textit{F. circinatum} proteins were also aligned to the pathogen-host interactions (PHI) database 4.2 [98] to identify potential pathogenicity and virulence factors using diamond. Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthology (KO) annotation was performed on all transcriptomes using GhostKOALA [99]. Read data and reference transcriptomes for \textit{P. patula} (BioProject PRJNA416698) and \textit{P. tecunumanii} (BioProject PRJNA416697) supporting the results of this article are available through the NCBI.

4.3. Mapping and Gene Expression Analysis

For both host species, the host and pathogen reference transcriptomes were combined (Figure S2). Read mapping and expression quantification, against the combined references, was performed using Kallisto 0.42.4 [100], with sequence bias correction and 40 bootstrap samples. The count data was imported into R 3.4.2 [101], using tximport 1.4.0 [102], for differential expression (DE) analysis. Fungal genes expressed at equal levels in inoculated and mock-inoculated samples, when normalizing against the full read set, were likely due to endophyte expression. Therefore, a high-confidence pathogen expressed gene set was produced to exclude potential endophyte contamination by performing a DE analysis using the full count data set (including both host and pathogen mapped reads) for each host (Figure S2). \textit{F. circinatum} genes significantly up-regulated in inoculated relative to mock-inoculated samples were considered high confidence expressed genes for each time point. Significantly down-regulated \textit{F. circinatum} genes, in inoculated relative to mock-inoculated samples, could represent potential endophyte genes and were discarded for downstream analysis. For pathogen DE analysis, \textit{F. circinatum} count data from both host species was combined. Pathogen expression data was removed from the count data for host DE analysis. Transcripts with less than 20 reads from at least three read libraries were classified as low expression transcripts and filtered out. DE testing was performed with DESeq2 1.18.1 [103] using a Wald test with Benjamini and Hochberg (BH) false discovery rate (FDR) correction \( p < 0.05, \text{Abs} \log_{2} \text{Fold-change} \geq 0.5 \). Biological pathways related to the differentially expressed genes (DEGs) were investigated using Mercator annotations with MapMan v3.5.1R2 [104], as well as KEGG orthology with KEGG mapper reconstruct pathway tool [105–107].

4.4. Gene Ontology Enrichment Analysis

DEGs for each comparison were divided into up- and down-regulated subsets (inoculated versus mock-inoculated for host transcripts; \textit{P. tecunumanii} vs. \textit{P. patula} for \textit{F. circinatum} transcripts, Figure S2). Significant enrichment of GO terms (BH FDR, \( p < 0.10 \)), relative to the transcriptome annotation for each species, was determined for each high confidence pathogen expressed gene set as well as the host and pathogen DEG subsets using GOSeq 1.28.0 [108].

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/9/315/s1.

Author Contributions: Conceptualization, E.V., E.S., A.M. and S.N.; methodology, E.V., J.W., E.S., and S.N.; software, J.W.; formal analysis, E.V.; investigation, E.V. and S.N.; resources, J.W., E.S. and S.N.; data curation, E.V.; writing—original draft preparation, E.V.; writing—review and editing, E.V., J.W., E.S., A.A. & S.N.; visualization, E.V.; supervision, J.W., E.S., A.A. and S.N.; project administration, E.V. and S.N.; funding acquisition, A.A and S.N.

Funding: This work was supported by the National Research Foundation (NRF) of South Africa Scarce Skills Doctoral Scholarship Programme (Grant ID: 97892), the NRF Bioinformatics and Functional Genomics Programme (Grant IDs: 86936, 97911) and a strategic grant from the Department of Science and Technology (DST) for the Tree Genomics Platform at the University of Pretoria. Further support was provided by Sappi, Mondi, York Timbers and Hans Merensky Foundation though the Forest Molecular Genetics (FMG) Programme with co-funding from the Technology and Human Resources for Industry Programme (THRIP, Grant ID: 96413). Opinions expressed, and conclusion arrived at are those of the author(s) and are not necessarily to be attributed to the NRF.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
References

1. Nirenberg, I.; O'Donnell, K. New Fusarium species and combinations within the Gibberella fujikuroi species complex. *Mycologia* **1998**, *90*, 434–458. [CrossRef]

2. Wingfield, M.J.; Hamerbacher, A.; Ganley, T.R.; Steenkamp, E.T.; Gordon, T.R.; Wingfield, B.D.; Coutinho, T.A. Pitch canker caused by *Fusarium circinatum*—A growing threat to pine plantations and forests worldwide. *Australas. Plant. Pathol.* **2008**, *37*, 319–334. [CrossRef]

3. Gordon, T.R.; Swett, C.L.; Wingfield, M.J. Management of *Fusarium* diseases affecting conifers. *Crop Prot.* **2015**, *73*, 28–39. [CrossRef]

4. Wingfield, M.J.; Coutinho, T.A.; Roux, J.; Wingfield, B.D. The future of exotic plantation forestry in the tropics and southern Hemisphere: Lessons from pitch canker. *S. Afr. For. J.* **2002**, *195*, 79–82. [CrossRef]

5. Earle, C.J. *Pinus Radiata*. The Gymnosperm Database. Available online: https://www.conifers.org/pi/Pinus_radiata.php (accessed on 24 September 2018).

6. Gordon, T.R.; Reynolds, G.J.; Wingfield, B.D.; Coutinho, T.A. Plasticity in plant-microbe interactions: A perspective based on the pitch canker pathosystem. *Phytoparasitica* **2017**, *45*, 1–8. [CrossRef]

7. Swett, C.L.; Reynolds, G.J.; Gordon, T.R. Infection without wounding and symptomless shoot colonization of *Pinus radiata* by *Fusarium circinatum*, the cause of pitch canker. *For. Pathol.* **2018**, *48*, e12422. [CrossRef]

8. Swett, C.L.; Gordon, T.R. First report of grass species (*Poaceae*) as naturally occurring hosts of the pine pathogen *Gibberella circinate*. *Plant Dis.* **2012**, *96*, 908. [CrossRef]

9. Swett, C.L.; Gordon, T.R. Endophytic association of the pine pathogen *Fusarium circinatum* with corn (*Zea mays*). *Fungal Ecol.* **2015**, *13*, 120–129. [CrossRef]

10. Hernandez-Escribano, L.; Iturrixta, E.; Elvira-Recuenco, M.; Berbegal, M.; Campos, J.A.; Renobales, G.; García, J.; Raposa, R. Herbaceous plants in the understory of a pitch canker-affected *Pinus radiata* plantation are endophytically infected with *Fusarium circinatum*. *Fungal Ecol.* **2018**, *32*, 65–71. [CrossRef]

11. Weete, J.D.; Abril, M.; Blackwell, M. Phylogenetic distribution of fungal sterols. *PLoS ONE* **2010**, *5*, e10899. [CrossRef]

12. Dupont, S.; Lemetais, G.; Ferreira, T.; Cavot, P.; Gervais, P.; Beney, L. Ergosterol biosynthesis: A fungal pathway for life or land? *Evolution* **2012**, *66*, 2961–2968. [CrossRef] [PubMed]

13. Liu, X.; Jian, L.; Yin, Y.; Ma, Z. Involvement of FgERG4 in ergosterol biosynthesis, vegetative differentiation and virulence in *Fusarium graminearum*. *Mol. Plant Pathol.* **2013**, *14*, 71–83. [CrossRef] [PubMed]

14. Gamir, J.; Darwiche, R.; Van’t Hof, P.; Choudhary, V.; Stumpe, M.; Schreiter, R.; Mauch, F. The sterol-binding activity of PATHOGENES-RELATED PROTEIN 1 reveals the mode of action of an antimicrobial protein. *Plant J.* **2017**, *89*, 502–509. [CrossRef] [PubMed]

15. Hodge, G.R.; Dvorak, W.S. Differential responses of Central American and Mexican pine species and *Pinus radiata* to infection by the pitch canker fungus. *New For.* **2000**, *19*, 241–258. [CrossRef]

16. Department of Agriculture, Forestry and Fisheries (DAFF). Report on Commercial Timber Resources and Primary Roundwood Processing in South Africa 2015/2016. Forestry South Africa. Available online: http://www.forestry.co.za/statistical-data/ (accessed on 27 June 2018).

17. Department of Water Affairs and Forestry (DWAF). Report on Commercial Timber Resources and Primary Roundwood Processing in South Africa 2001/2002. Forestry South Africa. Available online: http://www.forestry.co.za/statistical-data/ (accessed on 27 June 2018).

18. Crous, J.W. Post establishment survival of *Pinus patula* in Mpumalanga, one year after planting. *S. Afr. For. J.* **2005**, *205*, 3–11.

19. Mitchell, R.G.; Steenkamp, E.T.; Coutinho, T.A.; Wingfield, M.J. The pitch canker fungus, *Fusarium circinatum*: Implications for South African forestry. *South. For.* **2011**, *73*, 1–13. [CrossRef]

20. Roux, J.; Eisenberg, B.; Kanzler, A.; Nel, A.; Coetzee, V.; Kietzka, E.; Wingfield, M.J. Testing of selected South African *Pinus* hybrids and families for tolerance to the pitch canker pathogen, *Fusarium circinatum*. *New For.* **2007**, *33*, 109–123. [CrossRef]

21. Mitchell, R.G.; Wingfield, M.J.; Hodge, G.R.; Steenkamp, E.T.; Coutinho, T.A. The tolerance of *Pinus patula × Pinus tecunumanii*, and other pine hybrids, to *Fusarium circinatum* in greenhouse trials. *New For.* **2013**, *44*, 443–456. [CrossRef]

22. Liu, J.J.; Sturrock, R.N.; Benton, R. Transcriptome analysis of *Pinus monticola* primary needles by RNA-seq provides novel insight into host resistance to *Cronartium ribicola*. *BMC Genom.* **2013**, *14*, 884. [CrossRef]
23. Carrasco, A.; Wegrzyn, J.L.; Durán, R.; Fernández, M.; Donoso, A.; Rodriguez, V.; Neale, D.; Valenzuela, S. Expression profiling in *Pinus radiata* infected with *Fusarium circinatum*. *Tree Genet. Genomes* 2017, 13, 46. [CrossRef]  
24. Bagñaresi, P.; Biselli, C.; Ottù, L.; Urso, S.; Crispino, P.; Abbruscato, P.; Piffanelli, P.; Lupotto, E.; Cattivelli, L.; Valè, G. Comparative transcriptome profiling of the early response to *Magnaporthe oryzae* in durable resistant vs susceptible rice (*Oryza sativa*) genotypes. *PLoS ONE* 2012, 7, e51609. [CrossRef] [PubMed]  
25. Kawahara, Y.; Ono, Y.; Kanamori, H.; Matsumoto, T.; Itoh, T.; Minami, E. Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS ONE* 2012, 7, e94923. [CrossRef]  
26. Naidoo, S.; Visser, E.A.; Zwart, L.; du Toit, Y.; Bhadouria, V.; Shuey, L.S. Dual RNA-seq to elucidate the plant-pathogen duel. *Curr. Issues Mol. Biol.* 2012, 27, 127–142.  
27. Westerman, A.J.; Barquist, L.; Vogel, J. Resolving host-pathogen interactions by dual RNA-seq. *PLoS Pathog.* 2017, 13, e1006033. [CrossRef] [PubMed]  
28. Visser, E.A.; Wegrzyn, J.L.; Myburg, A.A.; Naidoo, S. Defence transcriptome assembly and pathogenesis related gene family analysis in *Pinus tecunumanii* (low elevation). *BMC Genom.* 2018, 19, 632. [CrossRef] [PubMed]  
29. Molnár, G.; Bancos, S.; Nagy, F.; Szkeres, M. Characterisation of BRH1, a brassinosteroid-responsive RING-H2 gene from *Arabidopsis thaliana*. *Planta* 2002, 215, 127–133. [CrossRef] [PubMed]  
30. Hou, B.; Lim, E.K.; Higgins, G.S.; Bowles, D.J. N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. *J. Biol. Chem.* 2004, 279, 47822–47832. [CrossRef]  
31. Poppenberger, B.; Fujioka, S.; Soeno, K.; George, G.L.; Vaistij, F.E.; Hiranuma, S.; Seto, H.; Takatsuto, S.; Adam, G.; Yoshida, S.; et al. The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. *Proc. Natl. Acad. Sci. USA* 2005, 102, 15253–15258. [CrossRef]  
32. Wang, D.; Pajerowska-Mukhtar, K.; Culler, A.H.; Dong, X. Salicylic acid inhibits pathogen growth in plants through repression of the auxin signalling pathway. *Curr. Biol.* 2007, 17, 1784–1790. [CrossRef]  
33. Wind, J.J.; Peviani, A.; Snel, B.; Hanson, J.; Smeekens, S.C. ABI4: Versatile activator and repressor. *Trends Plant Sci.* 2013, 18, 125–132. [CrossRef]  
34. Achard, P.; Cheng, H.; De Grauwe, L.; Decat, J.; Schoutteten, H.; Moritz, T.; Van Der Straeten, D.; Peng, J.; Harberd, N.P. Integration of plant responses to environmentally activated phytohormonal signals. *Science* 2006, 311, 91–94. [CrossRef] [PubMed]  
35. Resnick, J.S.; Wen, C.K.; Shockey, J.A.; Chang, C. Reversion-to-Ethylene Sensitivity1, a conserved gene that regulates ethylene receptor function in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 2006, 103, 7917–7922. [CrossRef] [PubMed]  
36. Arc, E.; Sechet, J.; Corbineau, F.; Rajjou, L.; Marion-Poll, A. ABA crosstalk with ethylene and nitric oxide in *Arabidopsis*. *Plant Phys.* 2013, 160, 1559–1567. [CrossRef] [PubMed]  
37. Wildermuth, M.C.; Dewdney, J.; Wu, G.; Ausubel, F.M. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 2001, 414, 562–565. [CrossRef] [PubMed]  
38. Delaney, T.P.; Uknes, S.; Vernooy, B.; Friedrich, L.; Weymann, K.; Negrotto, D.; Gaffney, T.; Gut-Rella, M.; Kessmann, H.; Ward, E.; et al. A central role of salicylic Acid in plant disease resistance. *Science* 1994, 266, 1247–1250. [CrossRef] [PubMed]  
39. Mano, Y.; Nemoto, K. The pathway of auxin biosynthesis in plants. *J. Exp. Bot.* 2012, 63, 2853–2872. [CrossRef] [PubMed]  
40. Seo, H.S.; Song, J.T.; Cheong, J.J.; Lee, Y.H.; Lee, J.W.; Hwang, I.; Lee, J.S.; Choi, Y.D. Jasmonic acid carboxyl methyltransferases: A key enzyme for jasmonate-regulated plant responses. *Proc. Natl. Acad. Sci. USA* 2001, 98, 4788–4793. [CrossRef] [PubMed]  
41. Yang, Y.; Xu, R.; Ma, C.J.; Vlot, A.C.; Klessig, D.F.; Pichersky, E. Inactive methyl indole-3-acetic acid ester can be hydrolysed and activated by several esterases belonging to the AtMES esterase family of *Arabidopsis*. *Plant Phys.* 2008, 147, 1034–1045. [CrossRef]  
42. Manosalva, P.M.; Park, S.W.; Forouhar, F.; Tong, L.; Fry, W.E.; Klessig, D.F. Methyl esterase 1 (StMES1) is required for systemic acquired resistance in potato. *MPMI* 2010, 23, 1151–1163. [CrossRef]  
43. Davièvre, J.M.; Achard, P. Gibberellin signalling in plants. *Development* 2013, 140, 1147–1151. [CrossRef]  
44. Wasternack, C.; Hause, B. Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in *Annals of Botany. Ann. Bot.* 2007, 111, 1021–1058. [CrossRef] [PubMed]
45. Naseem, M.; Kaltdorf, M.; Dandekar, T. The nexus between growth and defence signalling: Auxin and cytokinin modulate plant immune response pathways. *J. Exp. Bot.* **2015**, *66*, 4885–4896. [CrossRef] [PubMed]
46. Price, C.L.; Parker, J.E.; Warrillow, A.G.; Kelly, D.E.; Kelly, S.L. Azole fungicides—understanding resistance mechanisms in agricultural fungal pathogens. *Pest Manag. Sci.* **2015**, *71*, 1054–1058. [CrossRef] [PubMed]
47. Vale-Silva, L.A.; Coste, A.T.; Ischer, F.; Parker, J.E.; Kelly, S.L.; Pinto, E.; Sanglard, D. Azole resistance by loss of function of the sterol Δ5,6-desaturase gene (ERG3) in *Candida albicans* does not necessarily decrease virulence. *Antimicrob. Agents Chemother.* **2012**, *56*, 1960–1968. [CrossRef] [PubMed]
48. Yun, Y.; Yin, D.; Dawood, D.H.; Liu, X.; Chen, Y.; Ma, Z. Functional characterization of FgERG3 and FgERG5 associated with ergosterol biosynthesis, vegetative differentiation and virulence of *Fusarium graminearum*. *Fungal Genet. Biol.* **2014**, *68*, 60–70. [CrossRef] [PubMed]
49. Ayine-Tora, D.M.; Kingsford-Adaboh, R.; Asomaning, W.A.; Harrison, J.J.; Mills-Robertson, F.C.; Bukari, Y.; Sakyi, P.O.; Kaminta, S.; Reynisson, J. Coumarin antifungal lead compounds from *Millettia thomningii* and their predicted mechanism of action. *Molecules* **2016**, *21*, 1369. [CrossRef] [PubMed]
50. Koch, A.; Kumar, N.; Weber, L.; Keller, H.; Imani, J.; Kogel, K.H. Host induced gene silencing of cytochrome P450 lanosterol C14a-demethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 19324–19329. [CrossRef] [PubMed]
51. Simons, V.; Morrisey, J.P.; Latijnhouwers, M.; Csukai, M.; Cleaver, A.; Yarrow, C.; Osbourn, A. Dual effects of plant steroidal alkaloids on *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **2006**, *50*, 2732–2740. [CrossRef]
52. Yu, L.H.; Wei, X.; Ma, M.; Chen, X.J.; Xu, S.B. Possible inhibitory molecular mechanism of farnesol on the development of flocamazole resistance in *Candida albicans* biofilm. *Antimicrob. Agents Chemother.* **2012**, *56*, 770–775. [CrossRef]
53. Seyfthetl, C.; Tsuda, K. Salicylic acid signal transduction: The initiation of biosynthesis, perception and transcriptional reprogramming. *Front. Plant Sci.* **2014**, *5*, 697. [CrossRef]
54. Huot, B.; Yao, J.; Montgomery, B.L.; He, S.Y. Growth-defense tradeoffs in plants: A balancing act to optimize fitness. *Mol. Plant* **2014**, *7*, 1267–1287. [CrossRef] [PubMed]
55. Wojtaszek, P. Oxidative burst: An early plant response to pathogen infection. *Biochem. J.* **1997**, *322*, 681–692. [CrossRef] [PubMed]
56. Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **2005**, *43*, 205–227. [CrossRef] [PubMed]
57. Thomma, B.P.H.J.; Eggermont, K.; Penninckx, I.A.; Mauch-Mani, B.; Vogelsang, B.; Cammue, B.P.; Broekaert, W.F. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 15107–15111. [CrossRef] [PubMed]
58. Thomma, B.P.; Eggermont, K.; Tierens, K.F.; Broekaert, W.F. Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. *Plant Physiol.* **1999**, *121*, 1093–1102. [CrossRef] [PubMed]
59. Ferrari, S.; Plotnikova, J.M.; De Lorenzo, G.; Ausubel, F.M. *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J.* **2003**, *35*, 193–205. [CrossRef]
60. Mengiste, T. Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* **2012**, *50*, 267–295. [CrossRef]
61. Song, S.; Huang, H.; Gao, H.; Wang, J.; Wu, D.; Liu, X.; Yang, S.; Zhai, Q.; Li, C.; Qi, T.; et al. Interaction between MYC2 and ETHYLENE INSENSITIVE3 modulates antagonism between jasmonate and ethylene signalling in *Arabidopsis*. *Plant Cell* **2014**, *26*, 263–279. [CrossRef]
62. Grunewald, W.; Vanholme, B.; Pauwels, L.; Plovie, E.; Inzé, D.; Gheyssen, G.; Goossens, A. Expression of the *Arabidopsis* jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. *EMBO Rep.* **2009**, *10*, 923–928. [CrossRef]
63. Qi, L.; Yan, J.; Li, Y.; Jiang, H.; Sun, J.; Chen, Q.; Li, H.; Chu, J.; Yan, C.; Sun, X.; et al. *Arabidopsis thaliana* plants differentially modulate auxin biosynthesis and transport during defense responses to the necrotrophic pathogen *Alternaria brassicicola*. *New Phytol.* **2012**, *195*, 872–882. [CrossRef]
64. Zhou, N.; Tootle, T.L.; Tsui, F.; Klessig, D.F.; Glazebrook, J. *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* **1998**, *10*, 1021–1030. [CrossRef] [PubMed]
65. Falk, A.; Feys, B.J.; Frost, L.N.; Jones, J.D.G.; Daniels, M.J.; Parker, J.E. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3292–3297. [CrossRef] [PubMed]

66. Yang, Y.X.; Ahammed, G.J.; Wu, C.; Fan, S.Y.; Zhou, Y.H. Crosstalk among Jasmonate, Salicylate and Ethylene signalling pathways in plant disease and immune responses. *Curr. Protein Pept. Sci.* **2015**, *16*, 450–461. [CrossRef] [PubMed]

67. Makandar, R.; Nalam, V.; Chaturvedi, R.; Jeannotte, R.; Sparks, A.A.; Shah, L. Involvement of salicylate and jasmonate signalling pathways in *Arabidopsis* interaction with *Fusarium graminearum*. *MPMI* **2012**, *23*, 861–870. [CrossRef] [PubMed]

68. Liu, L.; Sonbol, F.M.; Huot, B.; Gu, Y.; Withers, J.; Mwimba, M.; Yao, J.; He, S.Y.; Dong, X. Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. *Nat. Commun.* **2016**, *7*, 13099. [CrossRef] [PubMed]

69. Staswick, P.E.; Tiryaki, I.; Rowe, M. Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *Plant Cell* **2002**, *14*, 1405–1415. [CrossRef] [PubMed]

70. Zhang, Z.; Li, Q.; Li, Z.; Staswick, P.E.; Wang, M.; Zhu, Y.; He, Z. Dual regulation role of GH3.5 in salicylic acid and auxin signalling during *Arabidopsis-Pseudomonas syringae* interaction. *Plant Physiol.* **2007**, *145*, 450–464. [CrossRef] [PubMed]

71. Chen, Y.; Shen, H.; Wang, M.; Li, Q.; He, Z. Saliciloyl-aspartate synthesized by the acyl-amido synthetase GH3.5 is a potential activator of plant immunity in *Arabidopsis*. *Acta Biochim. Biophys. Sin. (Shanghai)* **2013**, *45*, 827–836. [CrossRef] [PubMed]

72. Bari, R.; Jones, J.D.G. Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **2009**, *69*, 473–488. [CrossRef] [PubMed]

73. Kazan, K.; Mannes, J.M. Linking development to defense: Auxin in plant-pathogen interactions. *Trends Plant Sci.* **2009**, *14*, 373–382. [CrossRef] [PubMed]

74. Fu, J.; Wang, S. Insights into auxin signalling in plant-pathogen interactions. *Front. Plant Sci.* **2011**, *2*, 74. [CrossRef] [PubMed]

75. Visser, E.A.; Wegrzyn, J.L.; Steenkamp, E.T.; Myburg, A.A.; Naidoo, S. Combined de novo and genome guided assembly and annotation of the *Pinus patula* juvenile shoot transcriptome. *BMC Genom.* **2015**, *16*, 1057. [CrossRef] [PubMed]

76. Eckardt, N.A. Gibberellins are modified by methylation in planta. *Plant Cell* **2007**, *19*, 3–6. [CrossRef] [PubMed]

77. Wan, J.; Zhang, X.; Stacey, G. Chitin signaling and plant disease resistance. *Plant Signal. Behav.* **2008**, *3*, 831–833. [CrossRef] [PubMed]

78. Cosgrove, D.J. Loosening of plant cell walls by expansins. *Nature* **2000**, *407*, 321–326. [CrossRef] [PubMed]

79. Cantu, D.; Vicente, A.R.; Labavitch, J.M.; Bennett, A.B. Strangers in the matrix: Plant cell walls and pathogen susceptibility. *Trends Plant Sci.* **2008**, *13*, 610–617. [CrossRef]

80. Ding, X.; Cao, Y.; Huang, L.; Zhao, J.; Xu, C.; Li, X.; Wang, S. Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. *Plant Cell* **2008**, *20*, 228–240. [CrossRef]

81. Hrmova, M.; Farkas, V.; Lahnstein, J.; Fincher, G.B. A barley xyloglucan xyloglucosyl transferase covalently links xyloglucan, cellulose substrates, and (1,3;1,4)-β-D-glucans. *J. Biol. Chem.* **2007**, *282*, 12951–12962. [CrossRef]

82. Shinohara, N.; Sunagawa, N.; Tamura, S.; Yokoyama, R.; Ueda, M.; Igarashi, K.; Nishitani, K. The plant cell-wall enzyme AtXTH3 catalyses covalent cross-linking between cellulose and cello-oligosaccharide. *Sci. Rep.* **2017**, *7*, 46099. [CrossRef]

83. Miedes, E.; Vanholme, R.; Boerjan, W.; Molina, A. The role of the secondary cell wall in plant resistance to pathogens. *Front. Plant Sci.* **2014**, *5*, 358. [CrossRef]

84. Chini, A.; Fonseca, S.; Fernández, G.; Adie, B.; Chico, J.M.; Lorenzo, O.; García-Casado, G.; López-Vidriero, I.; Lozano, F.M.; Ponce, M.R.; et al. The JAZ family of repressors is the missing link in jasmonate signaling. *Nature* **2007**, *448*, 666–671. [CrossRef] [PubMed]
87. Gu, Y.Q.; Yang, C.; Thara, V.K.; Zhou, J.; Martin, G.B. Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell* 2000, 12, 771–786. [CrossRef] [PubMed]
88. Pirello, J.; Prasad, B.C.; Zhang, W.; Chen, K.; Mila, I.; Zouine, M.; Latché, A.; Pech, J.C.; Ohme-Takagi, M.; Regad, F.; et al. Functional analysis and binding affinity of the tomato ethylene response factors provide insight on the molecular bases of plant differential responses to ethylene. *BMC Plant Biol.* 2012, 12, 190. [CrossRef]
89. Cheng, Y.; Dai, X.; Zhao, Y. AtCAND1, a HEAT-repeat protein that participates in auxin signalling in *Arabidopsis*. *Plant Physiol.* 2004, 135, 1020–1026. [CrossRef] [PubMed]
90. Stotz, H.U.; Jikumaru, Y.; Shimada, Y.; Sasaki, E.; Stingl, N.; Mueller, M.J.; Kamiya, Y. Jasmonate-dependent and COI1-independent defense responses against *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*: Auxin is part of COI1-independent defense signalling. *Plant Cell Physiol.* 2011, 52, 1941–1956. [CrossRef]
91. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data. *Genome Biol.* 2014, 15, 550. [CrossRef]
92. Wingfield, B.D.; Steenkamp, E.T.; Santana, Q.C.; Coetzee, M.P.A.; Bam, S.; Barnes, I.; Beukes, C.W.; Chan, W.Y.; Nhlapo, N.; Beukes, A.; Baxter, J.; et al. Divergent functional responses to ethylene. *Plant Physiol.* 2004, 135, 448–459. [CrossRef] [PubMed]
93. Jones, P.; Binns, D.; Chang, H.Y.; Fraser, M.; Li, W.; McAnulla, C.; McWilliam, H.; Maslen, J.; Mitchell, A.; Nuka, G.; et al. InterProScan 5: Genome-scale protein function classification. *Bioinformatics* 2014, 30, 1236–1240. [CrossRef] [PubMed]
94. Buchfink, B.; Xie, C.; Huson, D. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 2015, 12, 59–60. [CrossRef] [PubMed]
95. Thines, B.; Katsir, L.; Melotto, M.; Niu, Y.; Mandaokar, A.; Liu, G.; Nomura, K.; He, S.Y.; Howe, G.A.; Browse, J. JAZ repressor proteins are targets of the SCF(CO1) complex during jasmonate signalling. *Nature* 2007, 448, 661–665. [CrossRef] [PubMed]
96. Huerta-Cepas, J.; Szklarczyk, D.; Forslund, K.; Cook, H.; Heller, D.; Walter, M.C.; Rattei, T.; Mende, D.R.; Abu-Ali, H.; Salgado, H.; et al. eggNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.* 2016, 44, D286–D293. [CrossRef] [PubMed]
97. Jiao, L.; Guan, Y.; Xie, Z.; Zhan, X.; Guo, Y.; Chen, G.; et al. GC skew in cultured tobacco cells. *Plant J.* 2000, 22, 571–579. [CrossRef] [PubMed]
98. Urban, M.; Pant, R.; Raghunath, A.; Irvine, A.G.; Pedro, H.; Hammond-Kosack, K.E. The Pathogen-Host Interactions database: Additions and future developments. *Nucleic Acids Res.* 2015, 43, D645–D655. [CrossRef] [PubMed]
99. Kanehisa, M.; Sato, Y.; Morishima, K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* 2016, 428, 726–731. [CrossRef] [PubMed]
100. Bray, N.L.; Pimentel, H.; Melsted, P.; Pachter, L. Near-Optimal RNA-seq quantification. *Nat. Biotechnol.* 2016, 34, 525–527. [CrossRef] [PubMed]
105. Kanehisa, M.; Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **2000**, *28*, 27–30. [CrossRef] [PubMed]

106. Kanehisa, M.; Sato, Y.; Kawashima, M.; Furumichi, M.; Tanabe, M. KEGG as a reference source for gene and protein annotation. *Nucleic Acids Res.* **2016**, *44*, D457–D462. [CrossRef] [PubMed]

107. Kanehisa, M.; Furumichi, M.; Tanabe, M.; Sato, Y.; Morishima, K. KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* **2017**, *45*, D353–D361. [CrossRef] [PubMed]

108. Young, M.D.; Wakefield, M.J.; Smyth, G.K.; Oshlack, A. Gene ontology analysis for RNA-seq, accounting for selection bias. *Genome Biol.* **2010**, *11*, R14. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).