**Transcriptome changes specifically associated with apple (*Malus domestica*) root defense
response during *Pythium ultimum* infection**

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ABSTRACT

RNA-seq technology was applied to identify the transcriptomic changes associated with apple root defense response to *Pythium ultimum* infection. Genes encoding homolog proteins with functions of pathogen detection such as chitin elicitor receptor kinase (CERK) and wall-associated receptor kinase (WAK) were among the differentially expressed apple genes. The biosynthesis and signaling of several plant hormones including ethylene, jasmonate and cytokinin were specifically induced in response to *P. ultimum* inoculation. Genes encoding enzymes of secondary metabolisms, cell wall fortification and pathogenesis related (PR) protein, laccase, mandelonitrile lyase and cyanogenic beta-glucosidase were consistently up-regulated in the later stages of infection.

Keywords: root defense response, transcriptomic change, soilborne necrotrophic pathogens, secondary metabolism, hormone regulation

Introduction

Apple replant disease (ARD) is caused by a complex of soilborne necrotrophic fungi (*Cylindrocarpon*, and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*), and at times it can be aggravated by the lesion nematode *Pratylenchus penetrans* [1-3]. *Pythium ultimum* is a primary component of ARD pathogen complex identified from orchard soil worldwide [1, 4-6]. When young trees are planted on a site that has a previous history of apple (or closely related species) cultivation, they develop disease symptoms ranging from mildly uneven growth to serious growth inhibition and even the death of trees [3, 7, 8]. The principal method for the control of ARD is pre-plant fumigation of orchard soils to eradicate ARD pathogens [9, 10] but the future availability of currently used fumigants could be restricted due to environmental concerns. Also, fumigation is not feasible after orchard establishment and the effect is short lived [11]. Alternative control methods, such as exploiting the interactions among microbial communities in orchard soil to promote plant health and minimize pathogen aggressiveness, has been shown to be promising for ARD management and can be effective in many situations [12, 13]. Host tolerance and/or resistance is an economically and environmentally attractive means of managing soilborne diseases in tree fruit production systems. Elucidating the molecular regulations that govern the defense responses in apple roots is a necessary step for efficiently exploiting the resistant trait through genomics-assisted breeding of resistant apple rootstock [14, 15].

Considerable progress has been made in elucidating the regulation networks during the interaction between plant and necrotrophic pathogens over the last decades, although the vast majority of the studies were carried out using foliar pathogens of model plant systems [16, 17]. It

is known that the biosynthesis and signaling of the plant hormones ethylene (ET) and jasmonate (JA) are critical in plant defense responses to necrotrophic pathogens [17, 18]. Pathways involved in the biosynthesis of pathogen-induced antimicrobial secondary metabolites (phytoalexins), such as the phenylpropanoid biosynthesis pathways, have long been associated with plant resistance [19, 20]. The co-regulation between these pathways is common during the defense response to invading pathogens. One example is JA signaling regulates the activation of transcription factor WRKY33 which leads to camalexin biosynthesis in Arabidopsis upon pathogen infection [21, 22]. The genotype specific variations on the dynamics of defense response may determine the outcome of the interaction between host and pathogen.

The investigation of the molecular interaction between plant root and soilborne pathogens has lagged considerably behind foliar pathosystems. The molecular defense response in apple roots in response to the infection by ARD pathogens has rarely been explored [23]. A transcriptomic approach can offer the genome-wide view of the molecular regulation networks underlying the defense responses in apple root tissue towards soilborne ARD pathogens. RNA sequencing (RNA-seq) technology, which simultaneously sequences the complementary DNAs of all transcript populations, has become a mainstay of transcriptomic analysis in last a few years; and newly developed bioinformatic software promises to offer high-resolution transcriptome profiles [24-26]. The aim of this study was to identify the specific transcriptomic changes in apple root tissue in response to infection by *P. ultimum*. The characterized transcriptome changes during apple root defense responses to *P. ultimum* inoculation should facilitate the identification of the key molecular components, which may differentiate the resistance and susceptibility among apple rootstock germplasm.

Materials and methods

Preparation of apple rootstock seedlings and Pythium inoculum and root tissue collection

Seeds derived from a cross between replant tolerant rootstock Geneva® 41 (G.41) [27] and replant susceptible rootstock Malling 26 (M.26) [28] were surface sterilized with 10% bleach and kept moist in a zip-lock plastic bag with 50 mg of Captan™ fungicide at 4°C for 6 weeks. After 6 weeks, seeds were planted in Sunshine™ potting mix soil (SUN GRO horticulture Ltd, Bellevue, WA) that had been pasteurized at 85°C for 12 hours. Seedlings were grown at 23°C and 95% humidity, under 12/12 hours light/dark conditions for 3 weeks.

The *Pythium ultimum* isolate used in this study was originally isolated from the roots of ‘Gala’/M26 apple grown at Moxee, WA. Inoculum of *P. ultimum* was prepared by cultivation in potato carrot broth (PCB: 20 g of carrots and 20 g of potatoes in 1 liter of medium with two drops of wheat germ oil added per liter of medium). The *P. ultimum* cultures were grown in the PCB in petri dishes at 22°C for 4-5 weeks. The resulting oospores and mycelium mat were ground in a blender for 30 sec. Spores and hyphal fragments were resuspended in 2% methyl cellulose and the inoculum was adjusted to a final concentration of approximately 2,000 colony forming units ml⁻¹.

The inoculation of seedlings with *P. ultimum* was performed by dipping the root system into the inoculum for 5 seconds and then planting treated seedlings into pasteurized Sunshine™ potting mix and water thoroughly. Control plants were mock inoculated with 0.5% methyl cellulose solution and then transplanted and maintained the same manner as pathogen infected plants. The inoculated seedlings were incubated in an environmental growth chamber at 23°C

and 95% humidity, under a 12/12 h light/dark photoperiod. Plants root tissues were harvested after 0, 1, 4, 8, 24, 48, 72, and 96 hours at which time root tissues were collected by excavating from soil, washing with water and flash-freezing in liquid nitrogen. Root tissue of twenty seedlings was collected and pooled for each time point per treatment. The experiment was repeated twice, and the pooled root tissues from the same time points after inoculation were used for RNA isolation and RNA-seq analysis. Frozen root tissues were stored at -80°C. The subsequent transcriptome data were analyzed by two-way comparisons, i.e. within tissue series (mock inoculated and *P. ultimum* infected), or between two tissue series at the same time point (Fig. S1, arrows).

High-throughput sequencing

The quantity and quality of isolated root RNA was examined using a Nanodrop ND-1000 (Thermo Scientific, Waltham, MA) and 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Purification of the poly-A containing mRNA and conversion to cDNA, TruSeq RNA sample preparation kit (Illumina, Inc., San Diego, CA) was employed. Prior to cDNA synthesis, 2 µg of total RNA was enriched for mRNA using oligo-dT attached magnetic beads. SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY) and first strand master mix from the TruSeq kit (Illumina, Inc., San Diego, CA) were used for first strand synthesis under the following conditions: 25°C for 10 min, 42°C for 50 min, 72°C for 15 min, and then held at 4°C. Second strand master mix from the TruSeq kit (Illumina, Inc., San Diego, CA) was used for second strand biosynthesis. RNA was fragmented by using divalent cations under elevated

temperature and short fragments were removed by using the AMPure XP beads. PCR-enrichment was performed under the following conditions: 98°C for 30 s; 15 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 30 s; 72°C for 5 min; and then held at 10 °C. The quality of libraries was checked with 2100 bioanalyzer and HS-DNA chip (Agilent Technologies, Inc., Santa Clara, CA). The 16 multiplex libraries (8 multiplex libraries per lane) were prepared in 2 lanes of Hi-seq 2000 (Illumina, San Diego, CA) for single-end sequencing of 101 bases without replication at the Center for Genome Research and Biocomputing in Oregon State University.

Reference mapping and RNA-seq analysis

The 15 sequence files containing 334.6 million raw reads in total were generated by the Illumina Hi-Seq 2000 instrument. The apple reference genome [29] *Malus domestica* v1.0 (122,107 contigs) was downloaded from the Genome Databases for Rosaceae (GDR, <http://www.rosaceae.org>). The genome sequence file consisted of 122,107 contigs. Data analysis was performed using CLC Genomics Workbench (CLC GW) v5.5.1 (CLCBio, Cambridge, Massachusetts). 332.6 million high-quality reads passed the quality score limit = 0.01 in CLC GW. These reads were used as input for large gap read mapping against reference genome Md-v1.0 using the CLC Large Gap Read Mapping plugin. CLC GW reference Mapper was run with high stringency settings, the minimum length fraction is 0.75 and the minimum similarity is 0.90. These two high stringent parameters were also used in large gap read mapping of sequences against *Malus domestica* v1.0. The limit for read unspecific match to *Malus domestica* v1.0 was set to 5. Revision of the reference transcriptome was conducted using the CLC Transcript

Discovery plugin, the parameters were set by default. For transcript discovery, the CLC Transcript Discovery plugin was applied, the parameters were set by default.

Differential gene expression analysis

In order to identify differences between the *P. ultimum* infected and mock inoculated root samples, RNA-seq analyses were performed for the *P. ultimum* infected (1, 4, 8, 24, 48, 72, 96 hpi) and mock inoculated root (0, 1, 4, 8, 24, 48, 72, 96 hpi) samples. Using the CLC Genomics Workbench software, the readings for each sample were separately mapped against the contigs with revised transcripts. The RNA-seq settings were the minimum length fraction is 0.75 and the minimum similarity is 0.90. The total gene count was set as gene expression value. Kal's statistical analysis test was used to compare gene expression levels between the *P. ultimum* infected and mock inoculated roots. The data were filtered to select the genes that satisfy FDR corrected $p < 0.001$, and with the detected fold change at or greater than 1.5. The identified DEs were saved for downstream analyses.

Functional annotation of candidate genes

Assigning InterPro domains and retrieving associated gene ontology (GO) terms describing biological processes, molecular functions, and cellular components [30] to genes was determined using InterProScan [31] and in-house scripts. The genes were assigned to their biologically relevant pathways using the KEGG pathway database through the KEGG

Automated Annotation Server (KAAS) [32]. Hyper geometric test on GO terms and KEGG pathway (Kyoto Encyclopedia of Genes and Genomes, <http://www.kegg.jp/>) terms were performed, the analysis of significant GO terms or KEGG pathway terms were identified with P-value < 0.05. The function annotation was obtained with the in-house scripts. TF database (<http://planttfdb.cbi.pku.edu.cn/download.php>) was searched using the BLAST algorithm [33]. BLASTX was conducted with the blastall (version 2.2.24). Only the best matches with an E-value of $\leq 1.0 \times 10^{-3}$ were recorded for transcripts.

Primer design

Forward and reverse primers for the genes used in this study were designed using web-based Primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and an IDT oligo analyzer (www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Where possible, an optimum annealing temperature of 60°C, a GC content of 40–60%, an amplicon length of 150–180 bp, and a primer length of 20 bp were applied. Multi-sequence alignment software ClustalW (www.ebi.ac.uk/clustalw) and BLAST (www.ncbi.nlm.nih.gov/BLAST/) were used to choose divergent regions at or close to 3' UTR (un-translated region) for gene-specific primer design.

RNA isolation and real-time qRT-PCR

The frozen tissue samples were ground to a fine powder in liquid nitrogen and total RNA was isolated following the modified method described by Gasic (2004) [34]. The total RNA was

quantified using a ND 1000 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA quality was verified by agarose gel electrophoresis. Total RNA was treated with DNase I (Qiagen, Valencia, CA) and then purified with RNeasy cleanup columns (Qiagen, Valencia, CA). Two µg of DNase-treated RNA was used to synthesize first-strand cDNA using SuperScriptTM II reverse transcriptase (Invitrogen, Grand Island, NY) and poly dT (Operon, Huntsville, AL) as the primer. The cDNA was diluted 20 times and 0.6 µL aliquot was used in a 15 µL quantitative PCR (qPCR) reaction mix: 0.45 µL SYBR Green I dye (Invitrogen, Grand Island, NY), 1X iTaq buffer (Biorad, Hercules, CA), 0.2 mM dNTP (Applied Biosystem, Waltham, MA), 2.5 mM MgCl₂, 0.3 units of iTaq DNA polymerase (Biorad, Hercules, CA) and 0.2 µM forward/reverse primer (IDT, Coralville, IA). Real-time qPCR amplification and detection was conducted using an iQ5 real-time qPCR detection system (Bio-Rad Lab, Hercules, CA) and the following protocol: cycle conditions of 3 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 59°C. Dissociation curves were run for all of the primers used in this study to determine the presence of any non-specific amplification. The relative gene expression was measured using the 0 hour root tissue as the calibrator. “No reverse transcriptase” and “no template” negative controls were included in PCR amplification. Each sample was represented by two independent total RNA isolations converted into two separate cDNAs. Each cDNA sample included two replicates for PCR reactions. Therefore, four separate PCR amplifications (four replicates) were performed on each sample. PCR amplification was carried out in triplicate in a 96 well plate. The target gene expression was normalized to that of the internal reference gene (*MdActin*) using the $2^{-\Delta\Delta CT}$ method (the comparative Ct method) [35, 36].

Results

Experimental design, reads processing and data analysis

RNA sequencing for 15 root samples produced 334.6 million raw reads in total, or an average 20 million of 100-bp single ended reads per sample. After quality control, these reads were mapped to the apple genome sequences consisting of 122,146 mega contigs [29]. Read mapping, transcript assembly and quantification were performed to identify the differentially expressed genes (DEGs) along the time points of infection process and between treatments, i.e. the mock inoculation and *P. ultimum* infection. Overall 90% of the reads were mapped to the draft apple genome sequences (Table 1). Among the mapped reads, 88% of them were ungapped reads and 75% of them were mapped to a specific location. RNA-seq data were deposited in GEO (Gene Expression Omnibus) with the accession number of GSE62103 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62103> at the NCBI website.

Two-way data analysis was performed for cross-examination of the specific transcriptomic changes associated with *P. ultimum* infection in apple root tissue (Fig. S1). 1. “M” and “P” datasets were generated by normalizing the transcript counts to those of sample before treatment or 0 hpi, within either mock inoculated or *P. ultimum* inoculated tissue series (1-96 hpi). M and P datasets demonstrate the transcriptome changes along the time course of infection (P) or those of mock inoculation (M). 2. “MP” dataset was generated by contrasting the normalized transcript counts between M (mock inoculation) and P (*P. ultimum* inoculation) datasets at each time point, i.e. the transcript levels in P dataset were calibrated by the value of the same transcript in M dataset. The resulting MP dataset represents the list of DEGs which are putatively specific to *P. ultimum* infection (Fig. 1C).

Identification of specific DEGs in response to *P. ultimum* infection

Among 23,000 discovered transcripts from root samples, up to 8% of them, depending on the time point and treatment, were identified as DEGs (using a cut off value of FDR 0.001). As shown in Fig. 1C, similar numbers of DEGs and a clearly decreasing trend were identified for both mock-inoculated and *P. ultimum*-inoculated apple root tissues at the early time points of 1, 4 and 8 hpi. Physiological perturbation from inoculation and seedling repotting process may cause the larger number of identified DEGs from both control and infected tissues at the earlier stages. Then a divergent trend of the number of identified DEGs was observed after 8 hpi. While the numbers of DEGs from the mock-inoculated tissues (M) continuously decreased, those from *P. ultimum* infected root tissues (P) increased considerably from 24 to 48 hpi, i.e. from 937 to 1457 DEGs. Correspondingly, the number of DEGs specific to *P. ultimum* infection in MP dataset steadily increased and peaked at 48 hpi. At earlier time points of 1, 4 and 8 hpi, the number of DEGs with double-digit or higher fold change is comparable between those exhibiting up- and down-regulated patterns. However, more DEGs demonstrated double-digit fold change and with up-regulated pattern in later stages (24-96 hpi) but only from the P dataset; and the opposite were observed from M dataset (Fig. S2).

Gene Ontology (GO) analysis: functional categorization of identified DEGs

Functional assignment and categorization for the identified DEGs was carried out by GO analysis using InterProScan and in-house scripts. Based on the side-by-side contrast between mock inoculation (M dataset) and *P. ultimum* infected tissues (P dataset), Fig. 2 demonstrated an

extensive changes of cellular activities in apple root due to the infection by *P. ultimum*, particularly in later stage of infection. The accumulative numbers of DEGs (of all time points) in MP dataset were categorized within three main categories: biological processes, molecular functions, and cellular components (Fig. S3-S5). The most enriched sub-categories of biological process are: oxidation-reduction process and metabolic process, followed by transport, transmembrane transport, isoprenoid biosynthetic process, response to biotic stimulus, aromatic amino acid family biosynthetic process, lipid metabolic process, glycolysis, and response to oxidative stress (Fig. S3). Other sub-categories including cation transport, cellulose biosynthetic process, drug transmembrane transport, lignin catabolic process also showed a significant increase in later stages (48-96 hpi) (Fig. 3). In the category of cellular component, most of the mapped DEGs were classified into a few sub-categories, including membrane, integral to membrane, apoplast, microtubule and protein complex (Fig. S4). Two sub-categories, membrane and integral to membrane, accounted for 75% of all DEGs assigned to the cellular component. This highlights the increased cross-membrane activities in apple root tissues in response to *P. ultimum* infection. The most enriched sub-categories under molecular function were those DEGs with the annotated function of oxidoreductase activity, catalytic activity, heme binding, electron carrier activity, iron ion binding, transporter activity, and ATPase activity (Fig. S5). Overall, considerable shift of cellular programs related to metabolic pathways, energy generation, transmembrane transport and oxidative imbalance were observed in apple root in response to the infection by *P. ultimum*.

KEGG pathway analysis: the role of secondary metabolisms

A systematic shift of cellular metabolism was observed between M and P datasets based on the KEGG pathway analysis on the identified DEGs (Fig. 3 and Table 3). Overall, up to 30% of the identified DEGs were mapped to various KEGG pathways. One of the most noticeable changes was an increased number of DEGs which mapped to multiple pathways related to secondary metabolism, such as phenylpropanoid biosynthesis, terpenoid backbone biosynthesis, flavonoid biosynthesis, stilbenoid, diarylheptanoid and gingerol biosynthesis, diterpenoid biosynthesis and bisphenol degradation (Fig. 3 and Table 3).

DEGs which encode enzymes functioning in multiple steps in phenylpropanoid biosynthesis pathway were exclusively identified from *P. ultimum*-infected tissue and with consistent up-regulation; and some of them are with double-digit fold increases in expression level (Table 3, Fig. S6 and Table S3). These enzymes included *phenylalanine ammonia-lyase* (MDP0000668828), *beta-glucosidase* (MDP0000175949 and MDP0000315857), *4-coumarate--CoA ligase* (MDP0000293578), *cinnamoyl-CoA reductase* (MDP0000322755), *trans-cinnamate 4-monooxygenase* (MDP0000225698, MDP0000602841, and MDP0000576346) and *coniferyl-aldehyde dehydrogenase* (MDP0000376347 and MDP0000438458). Multiple DEGs with the annotated function in flavanoid biosynthesis pathways were also mostly up-regulated, with a few exceptions of down-regulated expression patterns (Fig. S7 and Table S4). For example, *chalcone synthase* (MDP0000302905, MDP0000067565, MDP0000432621, and MDP0000686666), *chalcone isomerase* (MDP0000274127 and MDP0000759336), and *naringenin 3-dioxygenase* (MDP0000239947 and MDP0000166375) showed an up-regulated expression pattern only in *P. ultimum*-infected tissues. A DEG encoding *squalene monooxygenase* (MDP0000168437) functioning in sesquiterpenoid and triterpenoid biosynthesis pathway showed a 302 fold increase of transcript abundance. These two pathways are connected and squalene monooxygenase is in

the biosynthetic pathway of triterpenoid and other steroids (Fig. S10 and S11). Up to two dozen *ABC transporters*, which may function in delivering antimicrobial secondary metabolites to the extracellular location of host-pathogen interactions [37], were identified (data not shown). These results strongly suggested that the biosynthesis and transport of secondary metabolites is a critical component of apple root defense response to *P. ultimum* infection.

Identified DEGs functioning in other aspects of defense response

Many DEGs with the annotated functions of plant-pathogen recognition, reactive oxygen (RO) generation, phytohormone biosynthesis and signal transduction, and release of toxic compounds from preformed metabolites or phytoanticipins were identified (Table 2). The biological functions of the proteins encoded by these DEGs may not be readily categorized or highlighted by GO or KEGG Pathway Analysis.

Two DEGs encoding proteins homologous to *CERK1* (*a chitin elicitor receptor kinase 1*: MDP0000136494) and *WAK1* (*wall-associated receptor kinase*: MDP0000170906) were up-regulated from 24 to 72 hpi. A group of DEGs functioning in the anti-oxidative defense system such as those in the ascorbate-glutathione (AsA-GSH) cycle, which potentially prevents oxidative damage from accumulated reactive oxygen species (ROS), were dynamically regulated in the later stage of infection. These DEGs included those encoding *glutathione reductase* (GR) (MDP0000300208), *ascorbate peroxidase* (APX) (MDP0000199034 and MDP000024173) and flavonoid biosynthesis (*flavonol synthase/flavanone 3-hydroxylase*: MDP0000218810 and 2-oxoglutarate/*Fe (II)*-dependent dioxygenase: MDP0000128879) (Table 2, S1 and Fig. S8).

Multiple DEGs encoding proteins functioning in ET and JA biosynthesis and signaling were almost exclusively identified from *P. ultimum* infected tissues, such as *ACS* (*1-aminocyclopropane-1-carboxylate synthase*: MDP0000435100), *ACO* (*1-aminocyclopropane-1-carboxylate oxidase*: MDP0000195885 and MDP0000230092), *LOX* (*linoleate 9S-lipoxygenase*: MDP0000312397 and MDP0000423544), *ethylene receptor* (MDP0000920189), *JAZ* (*jasmonate ZIM domain-containing protein*: MDP0000387954, MDP0000871409 and MDP0000565690), and *ERF* (*ethylene-responsive transcription factor RAP2-3*: MDP0000413387) (Table 2, S2 and Fig. S9). Three *IAA-amino acid hydrolase* (MDP0000454027, MDP0000249564 and MDP0000622335), functioning to release the biologically active auxin from its conjugated form, were primarily up-regulated in response to *P. ultimum* infection. Two DEGs encoding *Gibberellin 2-beta-dioxygenase* (MDP0000245720 and MDP0000137705) showed up-regulated expression patterns. Noticeably, two DEGs for *cytokinin hydroxylases* (MDP0000305091 and MDP0000747755), which catalyzes the biosynthesis of trans-zeatin via the isopentenyladenine riboside 5'-monophosphate (iPRMP)-dependent pathway, showed 193 and 201 fold increases of transcript levels at 48 hpi, respectively (Table 2).

Several DEGs for pathogenesis-related (PR) proteins showed up-regulated expression patterns, including *glucan endo-1,3-beta-glucosidase* (*PR-2*: MDP0000147201 and MDP0000256573), *endochitinase* (*PR-4*: MDP0000655939 and MDP0000430546), and *thaumatin-like protein* (*PR-5*: MDP0000278380 and MDP0000317726). At least ten *laccases* encoding DEGs (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductases), which are involved in lignin metabolism, were identified, two of them (MDP0000291183 and MDP0000149146) showed double-digit up-regulated expressions. Seven DEGs for *mandelonitrile lyases* (EC 4.1.2.10), which catalyze the breakup of mandelonitrile to produce benzaldehyde and hydrogen

cyanide (HCN) [38] were identified. A double-digit fold increase in transcript levels was observed for one of them (MDP0000318256) from 48 to 72 hpi. A total of ten DEGs were identified as *cyanogenic beta-glucosidase*, and two of them (MDP0000047586 and MDP0000805281) showed 73.3 and 119.6 fold increases of transcript abundance at the peak response of 48 hpi, respectively. As the data indicated, extensive cellular metabolisms and signaling pathways, which are directly associated with plant defense response at various phases including pathogen detection, hormone signaling, biosynthesis of secondary metabolites and PR proteins and release of HCN, were specifically activated in response to the infection by *P. ultimum*.

Based pair-wise comparison of the transcriptome between *P. ultimum* inoculated and mock inoculated root tissues, the small numbers of DEGs were identified as pathogen infection specific DEGs at 1, 4 and 8 hpi. From MP dataset, 8, 58 and 29 DEGs were specifically regulated by *P. ultimum* infection, at 1, 4, and 8 hpi, respectively. The identities of the early activated genes were mostly related to the stress responses and oxidative metabolism. For example, several genes encoding glutathione S-transferase, monodehydroascorbate reductase, L-ascorbate peroxidase, dehydrin and heat shock 70 kDa protein were identified from at the early time points. Due to the small numbers of the identified DEGs, pathway analysis did not offer insightful conclusion (data not shown).

Transcription factors encoding DEGs specifically induced by P. ultimum infection

Many putative transcription factors (TFs) encoding DEGs were identified from P dataset (Fig. 4). The most enriched TF families included G2-like, LBD, NAC, WRKY and bHLH, which

presented double-digit numbers of DEGs identified from almost all time points. Other TF encoding gene families were also considerably enriched, such as B3, C2H2, C3H, ERF, FAR1, HD-ZIP, M-type, MYB, MYB-related, TCP and Trihelix. Several of these TF gene families, such as WRKY, NAC, bHLH and ERF, have been well characterized for their roles in plant defense responses in several foliar pathosystem [18].

Validation of the expression patterns of selected DEGs by qRT-PCR

The expression patterns for twelve *P. ultimum*-induced genes detected by RNA-seq based transcriptome profiling were validated by an independent approach of qRT-PCR (Fig. 5). Selected genes included those involved in various plant defense response processes including hormonal signaling such as *MdACS* and transcription factor *MdERF* and *MdWRKY33*, secondary metabolite biosynthesis such as *chalcon synthase* and *UDP-glucosyl transferase* and PR protein *MdCHIB*. Gene specific primer sets, reference numbers in the apple genome database and the RNA-seq data are shown on Table S7. Although different algorithms were used in quantifying their expression levels, a relatively comparable dynamic of gene expression patterns were observed by both approaches (Fig. 5).

Discussion

The results from this study, for the first time, reveal the dynamics of genome-wide defense responses in the root tissues of a perennial tree crop to *P. ultimum* infection. Genes functioning in several phases of plant defense response were specifically regulated which include

pathogen recognition, ROS (reactive oxygen species) and antioxidant metabolism, hormone biosynthesis and signaling, defense associated TF families and biosynthesis of antimicrobial secondary metabolites. It appeared that the observations based on foliar pathosystems are largely transferable to the root tissues - with some exceptions such as the potential role of cytokinin and the release of potent toxic HCN from phytoanticipins. The molecular framework derived from this study can serve as the foundation to further elucidate the roles of specific genes and pathways in root resistance to soilborne pathogens.

DEGs encoding protein homologous to CERK 1 and WAK1 are known to function in detection of pathogen presence by recognizing pathogen-associated molecular patterns (PAMP) [39, 40], and thereafter activation of the cellular defense system. Beside the receptor kinase, up to 53 other kinase encoding DEGs (data not shown) were also identified from P dataset indicating the kinase signaling cascade is critical to many defense associated cellular processes. DEGs related to oxido-reduction process constitute a key transcriptome change during *P. ultimum* infection (Fig. S3, S5, Table 2). Accumulation of ROS occurs in the early stages of plant-pathogen interaction; the enhanced activity of ROS scavenging systems such as ascorbate (AsA) and glutathione (GSH), which possess a strong reducing power and both act as a strong antioxidant as a radical scavenger [41], may play a critical role in protecting apple root tissues from high level ROS due to *P. ultimum* infection.

A sophisticated network of plant hormonal regulation is known to be a critical part of plant defense in response to the infection from necrotrophic pathogens [42, 43]. Specifically, a signaling network primarily regulated by ET and JA has been demonstrated to trigger a cascade of events that leads to enhanced plant resistance to necrotrophic pathogens [16, 42, 44-48]. ET/JA modulated defense responses during interaction between apple roots and *P. ultimum* were

also studied by a recent candidate gene based approach [23]. Our data demonstrated that many DEGs associated with ET/JA biosynthesis and signal transduction were activated during the interaction between apple roots and *P. ultimum*. On the other hand, the role of cytokinins (CK) in plant defense is poorly understood, although *Agrobacterium tumefaciens* modifies host CK biosynthesis to promote tumorigenesis [49] and CK also acts as a key factor in the development of clubroot disease caused by *Plasmodiophora brassicae* [50]. The strong induction of cytokinin hydroxylase encoding genes observed in this study suggests that cytokinin signaling may play a unique role in the defense response in apple roots defending against *P. ultimum*.

TFs play a crucial role in transcriptional reprogramming, and link the phytohormone signaling with downstream metabolic pathways which generate antimicrobial compounds and PR proteins [43, 51]. Members of WRKY, AP2/ERF, NAC, MYB and MYC/bHLH families regulate a defense-related gene expression [52, 53]. WRKYs can be activated by various factors including kinases (MAPKs), flg22, chitin and phytohormones (ABA and ET) [53] and are involved in various phytoalexin biosynthesis [20, 53]. ERFs are responsive to both pathogen-induced or exogenous applied ET and JA, and regulate downstream PR genes [23]. bHLH and MYC are known to play an essential role in JA signaling as repressors [53]. Therefore, TFs could be a pivotal factor for the outcome of plant-pathogen interactions among various genetic backgrounds [18, 54].

Metabolism reprogramming from normal growth and development processes to defense activation as a result of pathogen infection is critical to the survival of plant; for example, the production and transportation of antimicrobial metabolites to deter pathogen progression and to repair damaged tissues are known to be the integral parts of the plant defense mechanism [19, 20]. Both preformed antimicrobial compounds (phytoanticipins) and pathogen infection-induced

antimicrobial secondary metabolites (phytoalexins) have long been associated with plant resistance to fungal, oomycete and bacterial pathogens [55-57]. Cytological studies on the antimicrobial mechanisms of camalexin, produced by *Arabidopsis*, to *Botrytis cinerea* and *Alternaria brassicicola* indicated that phytoalexins cause the cessation of germ tube growth, disruption of plasma membrane integrity, cytoplasmic granulation, disruption of respiration, disorganization of mitochondria, and the induction of fungal apoptotic-like programmed cell death [58-60]. Identification of the specifically enriched metabolites due to infection should be a complementary approach to pinpoint crucial genes and branches of pathways. Moreover, although *de novo* biosynthesis of secondary metabolites is likely a principal defense action, the timely release of antimicrobial chemicals from preformed compounds could also be an integral part of the defense response exemplified by multiple DEGs with the annotated function of releasing HCN. Cyanogenic beta-glucosidase is responsible for the cleavage of cyanoglucosides and releases hydrogen cyanide (HCN). Due to its toxicity to aerobic organisms, HCN containing compounds such as cyanogenic glucosides have been referred as a phytoanticipins and play a role in plant defense against herbivores and pathogens [61]. HCN is also a co-product of ET biosynthesis pathway, and has been shown to contribute to plant resistance [62]. The data from this study unequivocally highlighted the role of “chemical warfare” between apple root and soilborne *P. ultimum*.

In addition to the role of anti-microbial metabolites in apple root defense, PR proteins and hydrolytic enzymes such as endo-1, 3-beta-glucosidase and endochitinase can disintegrate the cell wall of the necrotrophic pathogen; and the resulting oligosaccharides could also elicit plant defense reactions [63-65]. Thaumatin-like protein (PR-5) is able to disrupt plasma membrane and causes plasmolysis, as a result inhibits pathogen growth and development [63, 65,

66]. Most of the PR genes are inducible by plant defense-related phytohormones including ET and JA, and it was shown that an over-expression of PRs (thaumatin-like protein, beta-glucosidase or chitinase) enhanced plant resistance to fungal pathogens [23, 66].

RNA-seq technology is capable of covering the entire transcriptome. In this experiment, the raw reads that passed quality control were mapped to the un-annotated apple genome sequences consisting of a set of 122,146 contigs instead of mapping to non-redundant apple gene models [29]. About 90% of the reads were mapped to apple draft genome sequences which indicated a good quality RNA-seq dataset, and suggested the proper sample preparation prior to sequencing. The pair-wise comparison between M and P datasets at the same time points allow to remove the background noise and identify the DEGs specifically associated with the *P. ultimum* infection. Several factors can potentially affect the reliability and reproducibility of RNA-seq results including cDNA preparation, library construction, read mapping and assessment of transcript counts [67]. The fact that there are almost equivalent numbers of identified DEGs from the first three time points for both control (M) and infected tissues (P) suggested the comparability and consistency of the pooled root tissues of 20 seedlings. Using pooled root tissues, rather than those from a specific genotype, may be favorable in acquiring a more representing view of apple root defense responses, given the fact that almost no knowledge is available regarding the molecular defense response in apple root tissues. Single composite sample assay (the composite sample consisted of a pool of roots) and qRT-PCR confirmation that was used in this study has been routinely employed in RNA-seq based plant-pathogen interaction studies [68-71].

The possible molecular network underlying the defense response in apple root tissue to *P. ultimum* infection is illustrated in Fig 6. The detection of the pathogens presence by receptors

491 such as CERK1 homologous triggers extensive cellular changes such as reactive oxygen species
492 (ROS) burst and protein kinase activity. Subsequently, these cellular activities lead to
493 biosynthesis and signaling of plant hormones including ET, JA, CK and IAA [72-74]. Hormone
494 crosstalk and signal transduction pathways modulate the fine-tuning of transcriptional
495 reprogramming and metabolic redirection [18, 75, 76], through activating specific TFs . Then the
496 production of antimicrobial metabolites and proteins as well as the release of toxic chemicals
497 such as HCN from phytoanticipins could contribute to effective defense. It is conceivable that
498 the genotype-specific variations, such as the timing, intensity and duration of defense reactions
499 may lead to different dynamics of defense response, and ultimately determine the outcome
500 between apple root tissue and the invading pathogen.

501

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References

- [1] Jaffee BA, Abawi GS, Mai WF. Fungi associated with roots of apple seedlings grown in soil from an apple replant site. *Plant Dis* 1982;66:942–4.
- [2] Mazzola M. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. *Phytopathology*. 1998;88:930-8.
- [3] Jaffee BA, Abawi GS, Mai WF. Role of soil microflora and *Pratylenchus penetrans* in an apple replant disease. *Phytopathology*. 1982;72:247-51.
- [4] Mazzola M. Identification and pathogenicity of *Rhizoctonia* spp. isolated from apple roots and orchard soils. *Phytopathology*. 1997;87:582-7.
- [5] Tewoldemedhin Y, Mazzola M, Mostert L, McLeod A. *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. *Eur J Plant Pathol*. 2011;129:637-51.
- [6] Tewoldemedhin YT, Mazzola M, Botha WJ, Spies CFJ, McLeod A. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. *Eur J Plant Pathol*. 2011;130:215-29.
- [7] Mai WF, Abawi GS. Controlling replant disease of stone fruits in northeastern United States by preplant fumigation. *Plant Dis*. 1981;65:859-64.
- [8] Slykhuis JT, Li TSC. Responses of apple seedlings to biocides and phosphate fertilizers in orchard soils in British Columbia. *Can J Plant Pathol*. 1985;7:294-301.
- [9] Covey RP, Benson NR, Haglund WA. Effect of soil fumigation on the apple replant disease in Washington. *Phytopathology*. 1979;69:684–6.
- [10] Smith TJ. Orchard Update. Washington State Univ Coop Ext Bull September issue. Pullman, WA: Washington State University; 1995.
- [11] Mazzola M, Strauss SL. Resilience of orchard replant soils to pathogen reinfestation in response to *Brassicaceae* seed meal amendment. *Aspects Appl Biol*. 2013;119:69-77.
- [12] Mazzola M, Brown J, Zhao X, Izzo AD, Fazio G. Interaction of *Brassicaceous* seed meal and apple rootstock on recovery of *Pythium* spp. and *Pratylenchus penetrans* from roots grown in replant soils. *Plant Dis*. 2009;93:51-7.

539 [13] Mazzola M, Mullinix K. Comparative field efficacy of management strategies containing *brassica*
540 *napus* seed meal or green manure for the control of apple replant disease. *Plant Dis.* 2005;89:1207-13.

541 [14] Johnson WC, Aldwinckle HS, Cummins JN, Forsline PL, Holleran HT, Norelli JL, et al. The new
542 USDA-ARS/Cornell University apple rootstock breeding and evaluation program. *Acta Hort.*
543 2001;557:35-40.

544 [15] Fazio G, Aldwinckle HS, Robinson TL, Wan Y. Implementation of molecular marker technologies
545 in the apple rootstock breeding program in Geneva—challenges and successes. *Acta Hort.* 2011;903:61-8.

546 [16] Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens.
547 *Annu Rev Phytopathol.* 2005;43:205-27.

548 [17] Mengiste T. Plant immunity to necrotrophs. *Annu Rev Phytopathol.* 2012;50:267-94.

549 [18] Moore JW, Loake GJ, Spoel SH. Transcription dynamics in plant immunity. *Plant Cell.*
550 2011;23:2809-20.

551 [19] Grayer RJ, Kokubun T. Plant–fungal interactions: the search for phytoalexins and other antifungal
552 compounds from higher plants. *Phytochemistry.* 2001;56:253–63.

553 [20] Ahuja I, Kissen R, Bones AM. Phytoalexins in defense against pathogens. *Trends Plant Sci.*
554 2012;17:73–90.

555 [21] Gális I, Šimek P, Narisawa T, Sasaki M, Horiguchi T, Fukuda H, et al. A novel R2R3 MYB
556 transcription factor NtMYBJS1 is a methyl jasmonate-dependent regulator of phenylpropanoid-conjugate
557 biosynthesis in tobacco. *Plant J.* 2006;46:573-92.

558 [22] Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, et al. Antagonistic
559 interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene
560 expression and disease resistance in *Arabidopsis*. *Plant Cell.* 2004;16:3460-79.

561 [23] Shin S, Lv J, Fazio G, Mazzola M, Zhu Y. Transcriptional regulation of ethylene and jasmonate
562 mediated defense response in apple (*Malus domestica*) root during *Pythium ultimum* infection. *Hort Res.*
563 2014;1.

564 [24] Martin LB, Fei Z, Giovannoni JJ, Rose JK. Catalyzing plant science research with RNA-seq. *Front*
565 *Plant Sci.* 2013;4:66.

566 [25] Metzker ML. Sequencing technologies - the next generation. *Nat Rev Genet.* 2010;11:31-46.

567 [26] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Rev*
568 *Genet.* 2009;10:57-63.

569 [27] Fazio G, Aldwinckle HS, Robinson TL, Cummins J. (314) Geneva® 41: a new fire blight resistant,
570 dwarf apple rootstock. *HortScience.* 2005;40:1027.

571 [28] Wertheim SJ. Rootstock guide : apple, pear, cherry, European plum. Wilhelminadorp: Fruit Research
572 Station; 1998.

573 [29] Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, et al. The genome of
574 the domesticated apple (*Malus x domestica* Borkh.). *Nat Genet.* 2010;42:833-9.

575 [30] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: tool for
576 the unification of biology. *Nat Genet.* 2000;25:25-9.

577 [31] Zdobnov EM, Apweiler R. InterProScan – an integration platform for the signature-recognition
578 methods in InterPro. *Bioinformatics.* 2001;17:847-8.

579 [32] Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.*
580 2000;28:27-30.

581 [33] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol*
582 *Biol.* 1990;215:403-10.

583 [34] Gasic K, Hernandez A, Korban S. RNA extraction from different apple tissues rich in polyphenols
584 and polysaccharides for cDNA library construction. *Plant Mol Biol Rep* 2004;22:437-8.

585 [35] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative
586 PCR and the 2^{-ΔΔCT} Method. *Methods.* 2001;25:402-8.

587 [36] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc.*
588 2008;3:1101-8.

589 [37] Yazaki K. ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett.
590 2006;580:1183-91.

591 [38] Gross M, Jacobs GH, Poulton JE. A rapid and sensitive spectrophotometric assay for prunasin
592 hydrolase activity employing purified mandelonitrile lyase. Anal Biochem. 1982;119:25-30.

593 [39] Wan J, Zhang XC, Neece D, Ramonell KM, Clough S, Kim SY, et al. A LysM receptor-like kinase
594 plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell. 2008;20:471-81.

595 [40] Brotman Y, Landau U, Pnini S, Lisec J, Balazadeh S, Mueller-Roeber B, et al. The LysM receptor-
596 like kinase LysM RLK1 is required to activate defense and abiotic-stress responses induced by
597 overexpression of fungal chitinases in *Arabidopsis* plants. Mol Plant. 2012;5:1113-24.

598 [41] Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and
599 antioxidative defense mechanism in plants under stressful conditions. J Botany. 2012;2012:1-26.

600 [42] Pieterse CM, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SC. Hormonal modulation of
601 plant immunity. Annu Rev Cell Dev Biol. 2012;28:489-521.

602 [43] Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC. Networking by small-molecule
603 hormones in plant immunity. Nat Chem Biol. 2009;5:308-16.

604 [44] Shinshi H. Ethylene-regulated transcription and crosstalk with jasmonic acid. Plant Sci.
605 2008;175:18-23.

606 [45] Kazan K, Manners JM. Jasmonate signaling: toward an integrated view. Plant Physiol.
607 2008;146:1459-68.

608 [46] Okubara P, Paulitz T. Root defense responses to fungal pathogens: a molecular perspective. Plant
609 Soil. 2005;274:215-26.

610 [47] Ballare CL. Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. Trends
611 Plant Sci. 2011;16:249-57.

612 [48] Robert-Seilaniantz A, Grant M, Jones JD. Hormone crosstalk in plant disease and defense: more than
613 just jasmonate-salicylate antagonism. Annu Rev Phytopathol. 2011;49:317-43.

614 [49] Sakakibara H, Kasahara H, Ueda N, Kojima M, Takei K, Hishiyama S, et al. *Agrobacterium*
615 *tumefaciens* increases cytokinin production in plastids by modifying the biosynthetic pathway in the host
616 plant. Proc Natl Acad Sci USA. 2005;102:9972-7.

617 [50] Siemens J, Keller I, Sarx J, Kunz S, Schuller A, Nagel W, et al. Transcriptome analysis of
618 *Arabidopsis* clubroots indicate a key role for cytokinins in disease development. Mol Plant-Microbe
619 Interact. 2006;19:480-94.

620 [51] De Geyter N, Gholami A, Goormachtig S, Goossens A. Transcriptional machineries in jasmonate-
621 elicited plant secondary metabolism. Trends Plant Sci. 2012;17:349-59.

622 [52] Eulgem T. Regulation of the *Arabidopsis* defense transcriptome. Trends Plant Sci. 2005;10:71-8.

623 [53] Buscaill P, Rivas S. Transcriptional control of plant defence responses. Curr Opin Plant Biol.
624 2014;20:35-46.

625 [54] Birkenbihl RP, Somssich IE. Transcriptional plant responses critical for resistance towards
626 necrotrophic pathogens. Front Plant Sci. 2011;2:76.

627 [55] Hammerschmidt R. PHYTOALEXINS: What have we learned after 60 years? Ann Rev Phytopathol.
628 1999;37:285-306.

629 [56] VanEtten HD, Mansfield JW, Bailey JA, Farmer EE. Two classes of plant antibiotics: phytoalexins
630 versus "phytoanticipins". Plant Cell. 1994;6:1191-2.

631 [57] Bednarek P. Chemical warfare or modulators of defence responses – the function of secondary
632 metabolites in plant immunity. Curr Opin Plant Biol. 2012;15:407-14.

633 [58] Rogers EE, Glazebrook J, Ausubel M. Mode of action of the *Arabidopsis thaliana* phytoalexin
634 camalexin and its role in *Arabidopsis*–pathogen interactions. Mol Plant-Microbe Interact 1996;9:748-57.

635 [59] Sellam A, Iacomi-Vasilescu B, Hudhomme P, Simoneau P. *In vitro* antifungal activity of brassinin,
636 camalexin and two isothiocyanates against the crucifer pathogens *Alternaria brassicicola* and *Alternaria*
637 *brassicae*. Plant Pathol. 2007;56:296-301.

- [60] Shlezinger N, Minz A, Gur Y, Hatam I, Dagdas YF, Talbot NJ, et al. Anti-apoptotic machinery protects the necrotrophic fungus *Botrytis cinerea* from host-induced apoptotic-like cell death during plant infection. PLoS Pathog. 2011;7:e1002185.
- [61] Møller BL. Functional diversifications of cyanogenic glucosides. Curr Opin Plant Biol. 2010;13:337-46.
- [62] Seo S, Mitsuhashi I, Feng J, Iwai T, Hasegawa M, Ohashi Y. Cyanide, a coproduct of plant hormone ethylene biosynthesis, contributes to the resistance of rice to blast fungus. Plant Physiol. 2011;155:502-14.
- [63] Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, et al. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie. 1993;75:687-706.
- [64] Van Loon LC. Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol. 1997;103:753-65.
- [65] van Loon LC, Rep M, Pieterse CMJ. Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol. 2006;44:135-62.
- [66] Edreva A. Pathogenesis-related proteins: research progress in the last 15 years. Gen Appl Plant Physiology. 2005;31:105-24.
- [67] Labaj PP, Leparc GG, Linggi BE, Markillie LM, Wiley HS, Kreil DP. Characterization and improvement of RNA-Seq precision in quantitative transcript expression profiling. Bioinformatics. 2011;27:i383-91.
- [68] Gao L, Tu Z, Millett B, Bradeen J. Insights into organ-specific pathogen defense responses in plants: RNA-seq analysis of potato tuber-*Phytophthora infestans* interactions. BMC genomics. 2013;14:340.
- [69] Auer PL, Doerge RW. Statistical design and analysis of RNA sequencing data. Genetics. 2010;185:405-16.
- [70] Barakat A, Staton M, Cheng C-H, Park J, Yassin N, Ficklin S, et al. Chestnut resistance to the blight disease: insights from transcriptome analysis. BMC plant biology. 2012;12:38.
- [71] Fernandez D, Tisserant E, Talhinas P, Azinheira H, Vieira ANA, Petitot A-S, et al. 454-pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* reveals in planta-expressed pathogen-secreted proteins and plant functions in a late compatible plant-rust interaction. Mol Plant Pathol. 2012;13:17-37.
- [72] Hu X, Neill S, Yang Y, Cai W. Fungal elicitor Pep-25 increases cytosolic calcium ions, H₂O₂ production and activates the octadecanoid pathway in *Arabidopsis thaliana*. Planta. 2009;229:1201-8.
- [73] Govrin EM, Levine A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol. 2000;10:751-7.
- [74] Romeis T, Ludwig AA, Martin R, Jones JDG. Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. 2001;20:5556-67.
- [75] Grant MR, Jones JD. Hormone (dis)harmony moulds plant health and disease. Science. 2009;324:750-2.
- [76] Verhage A, van Wees SCM, Pieterse CMJ. Plant immunity: it's the hormones talking, but what do they say? Plant Physiol. 2010;154:536-40.

683

684 **Table 1.** Statistics of RNA-seq read mapping

		No. of Reads	Percentage
Mapped reads (90%)	Un-gapped read	265,450,835	88
	Gapped reads	35,170,165	12
	Total reads	300,621,000	
	Specific reads	224,382,784	75
	Non-specific reads	76,238,216	25
	Total reads	300,621,000	
Invalid mapped reads (2%)		6,148,087	2
Un-mapped reads (8%)		25,827,738	8
Total reads		332,596,825	

685

686

686 **Table 2.** Selected *P. ultimum* specific DEGs with annotated functions

Id in GDR	Annotated function	Fold change (hpi)						
		1	4	8	24	48	72	96
MDP0000678795	Cytochrome P450	ND	ND	ND	ND	53.2	46.2	41.9
MDP0000218810	Flavonol synthase/flavanone 3-hydroxylase	1.2	1.4	ND	ND	12.7	8.7	4.0
MDP0000128879	2-Oxoglutarate/Fe (II)-dependent dioxygenase	ND	ND	ND	ND	61.4	35.9	ND
MDP0000857724	NAD(P)-linked Oxidoreductase	ND	ND	ND	ND	48.4	21.7	32.2
MDP0000127630	Omega 3-fatty acid desaturase	ND	ND	ND	ND	16.4	12.6	ND
MDP0000156530	Omega 3-fatty acid desaturase	ND	ND	ND	ND	10.8	9.7	ND
MDP0000318256	Mandelonitrile lyase	ND	ND	ND	ND	29.6	27.8	29.0
MDP0000136494	CERK1, chitin elicitor receptor kinase 1	ND	ND	ND	ND	4.8	3.9	ND
MDP0000170906	Wall-associated receptor kinase	ND	ND	ND	ND	4.4	3.4	4.4
MDP0000195885	1-aminocyclopropane-1-carboxylate oxidase	ND	ND	ND	ND	20.4	28.9	17.8
MDP0000230092	1-aminocyclopropane-1-carboxylate oxidase	ND	ND	ND	ND	119.1	49.5	34.7
MDP0000435100	1-aminocyclopropane-1-carboxylate synthase	ND	ND	ND	ND	12.3	ND	ND
MDP0000312397	Probable linoleate 9S-lipoxygenase 5	ND	ND	ND	ND	14.4	8.4	ND
MDP0000423544	Probable linoleate 9S-lipoxygenase 4	ND	ND	ND	3.4	11.2	9.2	6.1
MDP0000920189	Ethylene receptor 2	ND	ND	ND	ND	3.0	2.1	ND
MDP0000413387	Ethylene-responsive transcription factor RAP2-3	ND	ND	ND	1.7	3.1	2.3	1.9
MDP0000305091	Cytokinin hydroxylase	ND	ND	ND	20.2	193.4	142.8	108.5
MDP0000747755	Cytokinin hydroxylase	ND	ND	ND	ND	201.4	170.3	ND
MDP0000454027	IAA-amino acid hydrolase	ND	ND	ND	2.3	4.2	3.2	2.7
MDP0000249564	IAA-amino acid hydrolase	ND	ND	ND	ND	3.8	3.3	ND
MDP0000622335	IAA-amino acid hydrolase	ND	ND	ND	2.3	2.6	2.5	2.0
MDP0000245720	Gibberellin 2-beta-dioxygenase	ND	ND	ND	ND	4.5	4.1	2.0
MDP0000137705	Gibberellin 2-beta-dioxygenase	ND	ND	ND	2.1	2.2	ND	ND
MDP0000668828	Phenylalanine ammonia-lyase	ND	ND	ND	ND	3.1	2.9	2.7
MDP0000302905	Chalcone synthase	ND	ND	ND	4.2	11.8	14.9	15.6
MDP0000067565	Chalcone synthase	ND	ND	ND	ND	24.2	19.9	16.0
MDP0000432621	Chalcone synthase	ND	ND	ND	5.5	37.2	30.9	33.4
MDP0000278380	Thaumatococcus-like protein	ND	ND	ND	ND	3.5	5.7	3.9
MDP0000317726	Thaumatococcus-like protein	ND	ND	ND	ND	3.9	6.1	4.8
MDP0000147201	Glucan endo-1,3-beta-glucosidase	ND	ND	ND	ND	2.0	ND	ND
MDP0000256573	Glucan endo-1,3-beta-glucosidase	ND	ND	ND	ND	2.9	6.1	6.9
MDP0000655939	endochitinase (PR-4)	ND	ND	ND	2.9	8.8	11.2	4.9
MDP0000430546	endochitinase (PR-4)	ND	ND	ND	ND	10.0	15.7	11.6
MDP0000199273	Beta-1,3-glucanase	ND	ND	ND	2.1	2.5	2.0	1.6
MDP0000291183	Laccases	ND	ND	ND	ND	10.6	8.0	ND
MDP0000149146	Laccases	ND	-1.5	-1.8	3.3	16.2	11.8	11.8
MDP0000318256	mandelonitrile lyase	ND	ND	ND	8.0	29.6	27.8	29.0
MDP0000903805	mandelonitrile lyase	ND	ND	ND	2.5	5.5	5.4	6.3
MDP0000047586	Cyanogenic beta-glucosidase	ND	ND	ND	9.2	73.3	47.4	19.5
MDP0000805281	Cyanogenic beta-glucosidase	ND	ND	ND	ND	119.6	54.0	65.7

MDP0000284104	Patatin	ND	ND	ND	4.2	16.6	18.3	19.6
MDP0000627477	Patatin	ND	ND	ND	ND	15.8	14.0	ND
MDP0000503940	Leucoanthocyanidin dioxygenase	ND	ND	ND	3.7	15.2	13.5	11.9
MDP0000566057	Leucoanthocyanidin dioxygenase	ND	ND	ND	ND	6.8	ND	ND
MDP0000788934	Leucoanthocyanidin dioxygenase	ND	ND	ND	1.9	2.3	1.9	1.8

687

688 The first column indicated the gene identification number from GDR (genome database for Rosaceae). ND: not
689 detected.

690 **Table 3.** DEGs mapped to various pathways of secondary metabolism

KEGG ID	Designated Pathways	No. of DEGs (hpi)						
		1	4	8	24	48	72	96
ko00900	Terpenoid backbone	0	0	0	9	11	10	0
ko00909	Sesquiterpenoid and triterpenoid	0	0	0	5	11	6	4
ko00940	Phenylpropanoid	0	0	0	0	25	21	19
ko00941	Flavonoid biosynthesis	0	0	0	8	17	17	14
ko00944	Flavone and flavonol	0	0	0	0	6	9	9
ko00945	Stilbenoid, diarylheptanoid and gingerol	0	0	0	0	8	10	8

691

692

FIGURE CAPTIONS

Fig. 1. Number of identified DEGs during the process of *P. ultimum* infection: The highest numbers of DEGs were identified at 1 hpi, for both mock- (M: solid line with filled circle) and *P. ultimum*-inoculated (P: dot line with open circle) tissues. The number of DEGs decreased in M dataset (dot line), a secondary peak was observed in P dataset (broken line). The solid line represents the DEGs after normalization of P dataset by M dataset or DEGs specific to pathogen infection. hpi: hour post inoculation.

Fig. 2. Major categories in GO biological process the identified DEGs were classified into. (A) Mock inoculation; (B) *P. ultimum* inoculation. X axis represents number of DEGs. Same scales on X axis are used for better visualization between treatments; Y axis indicated the selected GO categories. Time points are represented by different colors. Top 5 of the most enriched sub-categories (oxidation-reduction process, metabolic process, transport, transmembrane transport and isoprenoid biosynthetic process) were demonstrated in Fig. S3.

Fig. 3. Major pathways which the identified DEGs were mapped to using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis tools: (A) Mock inoculation; (B) *P. ultimum* inoculation. X axis represents number of DEGs. Same scales on X axis are used for better visualization between treatments; Y axis indicated the selected KEGG pathways. Time points are represented by different colors. See details in Fig S5-10.

Fig. 4. Identified DEGs which encode the transcription factors (TFs). Y axis represents numbers of DEGs that specifically responded to *P. ultimum* infection; Time points were represented by different colors.

Fig. 5. Validation of the expression patterns for genes selected from RNA-seq analysis by realtime qRT-PCR. Gene expression values were obtained by normalizing the values between *P. ultimum* inoculation and mock-inoculation control. Error bars represent the standard error for four independent experimental replicates.

Fig. 6. The illustration of molecular network underlying the defense response in apple root in response to the infection by *P. ultimum*.

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6