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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.

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1. 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 heath 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

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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.

2. Materials and methods

2.1. 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 h. Seedlings were grown at 23 °C and 95% humidity, under 12/12 h 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 L 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 s. Spores and hyphal fragments were resuspended in 2% methyl cellulose and the inoculum was adjusted to a final concentration of approximately 2000 colony forming units ml^{-1}.

The inoculation of seedlings with *P. ultimum* was performed by dipping the root system into the inoculum for 5 s 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 h 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).

2.2. 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.

2.3. 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.

2.4. 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