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Challenges and Progress in Evaluating Apple Root Resistance Responses to *Pythium ultimum* Infection

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Abstract

Due to the hidden nature of roots in the soils, it is more challenging to investigate their resistance traits and defense responses as compared to those of the aerial organs. At the same time, it is self-evident that root health is fundamental to a plant's entire life and productivity. It is also easily conceivable that root function, physiology, morphology, and architecture are constantly impacted by the complex soil environment including both biotic and abiotic factors. This report summarizes and updates the challenges and progress in evaluating resistance responses of apple root to infection from a necrotrophic oomycete pathogen, *Pythium ultimum*. Several obstacles impede the progress of investigating apple root resistance traits including the difficulties of direct and real-time evaluation and the lack of a continuous supply of apple plants for repeated infection assays. Systematic and detailed analyses were made possible by implementing a micropropagation procedure for continuously generating uniform apple plants for repeated infection assays. As a result, an elite panel of apple rootstock germplasm with distinct resistance levels was identified. These apple rootstock genotypes with well-defined resistance levels are the much-needed plant materials for subsequent genomics and transgenics analyses to define the functional roles of specific candidate genes. Careful microscopic examination revealed contrasting necrosis progression patterns between resistant and susceptible genotypes, which shed light on the potential mechanisms underlying resistance traits. Our continuing research will provide a clearer view regarding the genetic elements regulating resistance traits in apple roots to *P. ultimum* infection.

Keywords

Apple Roots, Defense Response, Resistance Trait, Micropropagation, Tissue

Culture, Microscopic Examination, *Pythium ultimum*

1. Introduction

It is self-evident that root health is fundamental to a plant's entire life and productivity. Root diseases including root rot and damping-off can occur in almost any plant species and the causal pathogens include fungi, oomycetes, and bacteria [1] [2] [3]. It is also easily conceivable that root function, physiology, morphology, and architecture are constantly impacted by the complex soil environment including both biotic and abiotic factors. However, the hidden nature of the root system often makes the study of root behaviors and responses more challenging compared to that of aerial organs. In terms of root diseases caused by soilborne pathogens, the early detection of physiological and pathological changes is the key to diagnosis and control [1] [4] [5]. Once disease symptoms are displayed on aerial parts of a plant, it is almost certain that the disease development in root tissues has unfortunately become advanced. Extra challenges exist for systematic and careful phenotypic assessment of root disease resistance traits as the genotype-specific difference in resistance response can be subtle or at the microscopic level at the early stage of pathogen infection.

The effective control of soilborne pathogens is significantly hindered by the poor accessibility of root systems in soil and the persistence of pathogens due to their ability to form survival structures (e.g., oospores, chlamydospores, and sclerotia) [6]. Several control methods have been applied to manage plant root diseases [1] [3] [7]. These methods include cultural measures such as crop rotation and field sanitation, biological measures such as soil amendment and use of biocontrol agents and chemical measures such as soil fumigation and application of other pesticides. Additionally, development and deployment of resistant cultivars are commonly viewed as a more favorable strategy for managing root diseases [8] [9] [10] [11]. In orchard systems management of soilborne diseases has relied heavily on soil fumigation using wide-spectrum biocides. Use of these "kill-everything" chemical reagents generates only a short-term effect on pathogen suppression, yet raises serious environmental concerns, and demolishes the soil microbiome. Biocontrol and cultural measurement to manage plant soilborne disease, such as soil amendment with specific organic materials, can be a part of integrated management approaches, but the efficacy is less predictable. It is well accepted that the development and deployment of resistant varieties can offer a durable, environmentally beneficial, and cost-effective strategy for managing soilborne diseases. However, challenges and obstacles exist before such genetic power can be realized for maximized exploitation of host resistance and for managing the soilborne disease. Conventional breeding of a resistant variety is a long-term and resource-demanding endeavor, particularly for a perennial tree fruit crop like apple. A better understanding of the genetic control over resistance traits, combined with genetics-based predictive tools, can greatly assist

the breeding process [8] [9]. However, systematic, detailed, and reliable characterization of resistance response in apple roots is essential to unravel the underlying genetic regulation of disease resistance in apple roots.

Multiple obstacles must be overcome to acquire high-quality phenotypes and detailed resistance responses among apple rootstock genotypes. At the most basic level, the hidden nature of root systems in soil significantly limits the accessibility of roots for a non-invasive, non-destructive evaluation of real-time symptom development. Innovative methods or monitoring systems such as transparent pots or other monitoring systems like rhizotrons have been developed to track and document root growth response and architectural patterns [12]. However, thorough evaluation of resistance response in young apple roots, such as closeup examination of necrosis progression patterns, can be prohibitively difficult due to the small stature of individual young (feeder) roots [13]. Secondly, root development such as lateral root initiation and differentiation of root branches is undetermined and irregular. This is in direct contrast to plant leaves where a fixed position of a leaf along a shoot is in general more comparable between plants in terms of their physiology and function. Lack of comparability between root systems between individual plants, or root branches within a root system, can be a practical barrier for consistent sampling and detailed analysis. In other words, the irregular patterns of root initiation, growth, and differentiation as well as their plasticity in response to surrounding environmental factors make it more challenging for detailed analysis of resistance response between apple rootstock genotypes.

Several unique challenges impede the investigation of resistance responses in the roots of apple as a woody *Rosaceae* species. The reproduction of apples is self-incompatible or outcrossing in nature, and the apple genome has a high-level heterozygosity [14] [15]. As a result, each seed in a fruit represents a unique genetic identity. In other words, seed germination cannot produce apple plants with identical genetic backgrounds. Meanwhile, repeated infection assays are essential for a systematic and reliable evaluation of apple root resistance response. As such, the continuous availability of apple plants with identical genetic backgrounds and clean or unchallenged roots becomes a practical challenge for studying apple root resistance responses. Perhaps the restricted availability of genetically uniform apple plants is one of the primary reasons that the focused study of apple root resistance response has been lacking until recently [8] [16] [17] [18] [19] [20]. At the same time, it is well acknowledged that high-quality phenotype data and in-depth characterization of resistance responses are the prerequisites for any meaningful molecular and genetic analysis for targeted traits [21]. Therefore, a constant supply of apple plants with uniform genetic backgrounds and comparable physiology by implementing a tissue culture procedure is fundamental for this study, although tissue culture procedure is notoriously time-consuming, laborious, and tedious.

Plant tissue culture is a century-old technique that allows vegetatively propagating clean and healthy plants based on the concept of totipotency [22]. With a

synchronized micropropagation procedure, apple plants of multiple apple rootstock genotypes can be generated simultaneously for comparative evaluation of their resistance responses. In addition, using apple plants with equivalent age and physiology from this process facilitates the identification of potentially subtle variations of resistance responses between genotypes [23]. The small size of four-week-old apple plants offers the advantage of easy handling in lab and greenhouse settings. This report attempts to summarize the challenges and progress on assessing the genotype-specific resistance responses in apple root to infection from a necrotrophic soilborne filamentous oomycete pathogen *P. ultimum*, which is a major component in a pathogen complex inciting apple replant disease (ARD). In short, three pillars in this study form a necessary platform for carrying out this study: implementing the apple micropropagation procedure, standardizing an effective inoculation method, and exploring a variety of methodologies to define the root responses at various aspects of infection from *P. ultimum*.

2. Plant Materials, Their Micropropagation and In-Soil Acclimation of Generated Root System

The primary plant materials are progeny from a cross between two elite apple rootstock varieties, "Ottawa 3" and "Robusta 5" (O3R5 for simplicity). Both parents have a strong background of wild apple germplasm [24] [25]. The progeny of the O3R5 cross was known for segregating multiple agronomical traits including dwarfism, resistance to apple fire blight and powdery mildew [26] [27] [28]. The commercialized ARD tolerant germplasm G.935® was selected from the O3R5 cross population [29], which suggested segregation for ARD resistance traits. Following a pilot study of testing and adjusting the phenotyping protocol using two apple rootstock genotypes of G.935 and Bud 9 [30], more than 90 genotypes from the O3R5 cross population were assessed for their resistance responses to *P. ultimum* infection under controlled experimental conditions.

The tissue culture-based micropropagation procedures for generating apple plants with defined genetic backgrounds and equivalent ages were described previously [30]. Briefly, 4 - 6 weeks are required for shoot propagation and another 4 weeks for root induction and elongation. After a sufficient root system (normally at 4 weeks of root elongation for most genotypes) has been developed, plants are transferred to pots typically containing autoclaved Sunshine™ potting mix (SUN GRO Horticulture Ltd, Bellevue, WA) or other types of soil media for one week of in-soil acclimation before pathogen infection assays. This step of in-soil acclimation is considered critical for roots generated from tissue culture medium transitioning to real soil conditions, allowing root tissues to further differentiate and fully express their inherent resistance traits [30] [31]. To minimize transplanting effects on plants during roots adapting from more conducive conditions in tissue culture medium to potting soils, a transparent 7" Vented Humidity Dome (Greenhouse Megastore, Danville, IL) was used to cover the tray

containing pots for at least 48 hr. The temperature in the growth room was approximately 22˚C ± 1˚C at night and 25˚C ± 1˚C during daytime with 12 hr light/12 hr dark photoperiod.

3. Pythium ultimum Inoculum Preparation, Root-Dipping Inoculation, and Types of Soil Medium

On the pathogen side, *Pythium ultimum* is a fast-growing oomycete, a type of eukaryotic organism that superficially resembles filamentous fungi but differs phylogenetically [32]. The genus *Pythium*, which consists of over 100 species, is ubiquitously distributed and capable of long-term survival in soil by producing thick-walled oospores. Germination of phytopathogenic *Pythium* spp. oospores initiate infection of seeds or roots resulting in damping off or root rot, which leads to plant wilting, reduced yield, and mortality on many economically important crops [32] [33]. *P. ultimum* is considered one of the most significant plant oomycete pathogens and was recently voted one of the top 10 plant oomycete pathogens by experts in the research field [32]. *P. ultimum* has been identified as a major component in the pathogen complex that incites ARD, causing stunted growth or death of newly planted trees in replant sites [34]. Incidentally, the fast-growing nature of *Pythium* hyphae allows the pathogen to spread to the whole root system overnight, adding difficulties for assessing the genotype-specific resistance response. For example, it is basically impossible to perform a localized inoculation for investigating tissue-specific defense responses. Plus, it is also difficult to select specific root segments, which have equivalent pathogenesis processes between plants of different genotypes, for time-defined microscopic observations of resistance response at the tissue level.

The *P. ultimum* isolate used in this study was originally isolated from the roots of "Gala"/M26 apple grown at Moxee, WA, USA. The inoculum was prepared as previously described [30]. Briefly, *P. ultimum* was cultivated in autoclaved potato-carrot broth (20 g of carrots and 20 g of peeled potatoes per L of water, boiled for 30 min, with two drops of wheat germ oil added per L) in 9-cm Petri dishes at 22˚C for 4 - 6 weeks [35]. Oospores and mycelium from the resultant hyphae mats were collected and ground in 2% methylcellulose solution using a household electric blender for 30 s. The oospores and hyphal fragments were resuspended in 2% methylcellulose to give a final concentration of approximately 2000 oospores per mL. Several inoculation methods were tested including 1) drenching soil with inoculum solution around apple plants in pots; 2) pre-mixing pathogen inoculum into potting soil and 3) dipping roots into inoculum solution. Root dipping method, *i.e.*, dipping the root system in the inoculum solution for 5 s, was eventually chosen as the inoculation method for its simplicity and consistency between infection assays. Inoculated plants and mock-inoculated plants were immediately transplanted into autoclaved Sunshine^{TM} potting mix in pots, watered thoroughly and maintained under the same conditions in an environmental growth room.

Several types of soil media were used during this study (also referring to Figure 1 legends). Orchard soils were initially adopted with the assumption that this type of medium is the natural physiochemical environment where interactions between apple root and pathogen occur (Figure 1(a)). However, a long-term supply of orchard soil with consistent biological, physical, and chemical properties can be a logistical issue. Subsequently, for most of the initial survey of genotype-specific resistance responses the commercially available Sunshine™ potting mix was used (Figure 1(b)). Nevertheless, the potting mix contains relatively rich organic materials and an undefined collection of microflorae. Plus, some microorganisms could not be eliminated by repeated autoclaving processes. Therefore, in the later stages of this study, particularly for those studies related to the microscopic examination of apple root response to *P. ultimum* infection, construction sand was used as the supporting medium (Figure 1(c)). This type of simplified supporting medium is fitting for investigating the early defense response at the tissue level which requires a relatively short growing period (normally less than a week). Using sand as a soil medium also adds the benefit that the roots stay relatively clean without organic debris attached or embedded to the root surface, which facilitates microscopic examination and documentation. In addition, even a "soilless" or hydroponic system was tested but was determined not to be an optimal system for dealing with larger numbers of plants or for a longer-term evaluation period (Figure 1(d)). In each infection assay, a mock-inoculation of control plants (with 2% methylcellulose solution without pathogens) was conducted prior to the pathogen-inoculation procedure to avoid unintentional cross-contamination of pathogens to control plants.

4. Systematic Characterization of Apple Root Resistance Responses

4.1. Plant Survival Rate as an Initial Indicator of Overall Resistance Response to *P. ultimum* Inoculation

At the early phase of the phenotyping project, the primary goal is to survey the range or levels of apple root resistance among tested O3R5 genotypes to *P. ultimum* infection. For this purpose, the average plant survival rate from repeated infection assays was used as the primary indicator of resistance level for a genotype. The average plant survival rates, with at least three independent infection assays, were acquired for 65 O3R5 genotypes, out of 90 genotypes initially included. Typically, through the synchronized micropropagation process, 4 - 8 genotypes with minimal 20 - 25 plants per genotype were included per infection event. The primary limitation is the capacity to generate enough plants from this tedious and laborious tissue culture process. Both pathogen-inoculated and mock-inoculated control plants were closely observed 2 - 3 times within the first 48 hpi (hours post inoculation) to discern possible responses either from transplant shock or pathogenic factors. The timing and severity of symptoms, including leaf wilting, partial browning from leaf edges, or whole-plant mortality, were documented at 3, 7, 10, 14 and 28 dpi (days post inoculation). For simplicity,

Figure 1. Phenotypic survey on resistance levels of O3R5 progeny at different phases and using various pots and soil media. (a) An image shows the setup for the early phase of phenotypic study, with three plants per 9-inch pots filled with autoclaved orchard soils. In each panel (of three), left columns shows mock-inoculated plants, and the right column shows plants inoculated with *P. ultimum* at 14 dpi. (b) In the later stage of the study, 5-inch pots were used with three plants per pot filled with autoclaved commercial potting mix. An image shows the distinct response of two genotypes at 14 dpi. For both trays, the plants in two pots at the left column were mock-inoculated plants, the rest of 24 plants in 8 pots were pathogen-inoculated. The plants on the top tray demonstrate a typical resistant genotype, and the plant in tray at the bottom shows a typical susceptible genotype. (c) An example showing the contrasting response from *P. ultimum* infection. The square pots were used with 5 plants per pot filled with autoclaved construction sands. The single pot at the top left of the tray shows the mock-inoculated plants, while the other three pots were plants inoculated with *P. ultimum*. (d) An image showing the test of a soil-less method for carrying out an infection assay to assess resistance response between apple rootstock genotypes to *P. ultimum* infection.

those genotypes showing consistent survival rates below 30% were designated as "susceptible", and those higher than 80% as "resistant" [17]. The final survival rate was assigned based on the observation at 28 dpi (days post inoculation), but the genotype-specific plant mortality was often stabilized at 7 dpi. Some genotypes with extreme susceptibility such as O3R5-47 and O3R5-132 exhibited observable wilting symptoms as early as 3 dpi or earlier [17] [31]. In rare cases, the infected plants with initial partial wilting symptoms at 3 dpi recovered and then survived until final evaluation at 28 dpi. The pots containing mock-inoculated plants were placed in separate trays to avoid the potential spread of the fast-growing *Pythium* hyphae through excess water beneath the pots in a tray. It is worth noting that the number of mock-inoculated plants (as control) was reduced, from half of the available plants initially to only 5 - 6 plants at a later

stage, based on the experience of handling apple plants (**Figures 1(a)-(c)**). This is intentional to put more plants subject to pathogen infection treatment, therefore, to enhance the reliability of the assessed resistance level for a tested genotype. This is also because producing apple plants from tissue culture procedure is still a huge burden throughout this study. Based on these initial phenotypic data, the genotypes with defined resistance or susceptibility were selected for subsequent focused investigation including the whole root system, biomass reduction, necrosis progression patterns or resistance responses at cellular or subcellular levels.

4.2. Assessment of Growth Suppression and Biomass Reduction Using a Glass Box

While plant survival rate is an essential indicator of resistance level assigned to a tested genotype, the stunted growth among the surviving plants also demonstrated variable degrees of resistance under pathogenic pressure. Levels of suppressed growth may shed light on the resistance mechanism associated with a specific genotype. The growth inhibition of roots and shoots was assessed for selected genotypes using a large glass box with the dimension of 30 w × 45 h × 10 d cm (**Figure 2(a)**). Root growth behaviors such as reduced branching and shortened elongation can be visualized and monitored for up to two months. Plant roots were aligned between the glass wall and a paper towel, then the rest of the space was filled with potting soil. The majority of the root system can be observed and documented by collecting time-lapse images to compare their growth, branching patterns and necrosis development between treatments and genotypes (**Figure 2(a)**, **Figure 2(b)**). Data of plant biomass reductions were evaluated for selected genotypes with distinct resistance responses at 28 dpi. The severity of growth suppression and biomass reduction can be inferred from analyzing the series of images during the time in the glass boxes, as well as by measuring fresh weight per plant at the end of the experiment. Plants were carefully removed from glass boxes, and soil was gently rinsed off root tissues under tap water. Individual plants were wrapped in moist paper towels before weighing the shoot and root separately. Leaves were separated and laid out on white paper and root branches were separated and dispersed in a petri dish before taking images using a Canon D35 camera (**Figures 2(c)-(e)**). Total leaf area, stem lengths and total root lengths were measured by processing the acquired images using the publicly available software ImageJ (https://imagej.nih.gov/ij/). For most susceptible genotypes, biomass reduction (for either root or shoot) showed statistically significant differences at 28 dpi, when the values of the surviving plants from *P. ultimum* inoculation were compared with those of mock-inoculated control plants [17]. Due to the variation of lateral root initiation and growth rate, which is difficult to predict or control, between individual plants generated from tissue culture procedure, it is relatively common to observe large standard deviations for this type of assay. Nevertheless, the data on root system inhibition and biomass reduction added information to the plant survival rates and increased our confidence in assigning the resistance levels to an individual tested genotype.

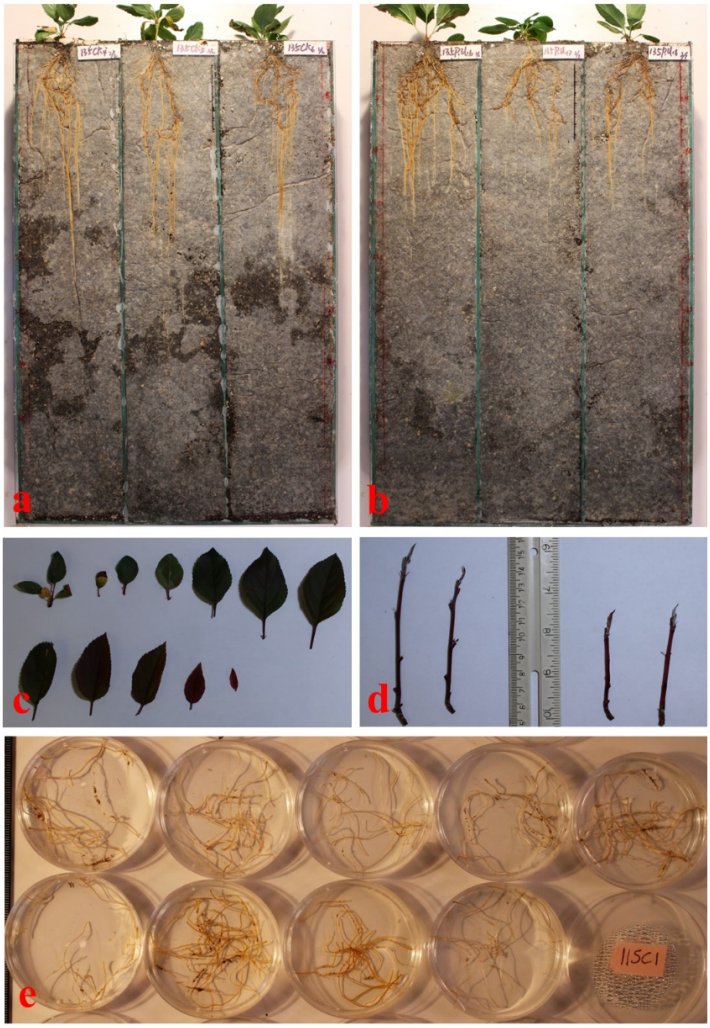

Figure 2. Genotype-specific growth suppression and biomass reduction using a glass-box base observation and measurement. (a) an image shows mock-inoculated, and (b) an image shows *P. ultimum* inoculated O3R5-135 plants in a large glass-box at 14 dpi. Most root systems were exposed for observation and documentation for their growth behavior during 8-week period. (c) and (d) show the assembly of separated leaves and stems for measurement of total area and length between different treatments and genotypes. (e) root branches in petri dishes for measurement of total root length for individual plants, using software ImageJ (https://imagej.nih.gov/ij/) on captured images at the end of the experiment.

4.3. Microscopic Features of Genotype-Specific Necrosis Patterns

To further understand the detailed and real-time resistance responses at the tissue and cellular levels, microscope-assisted observation became essential. For example, the timing and intensity of pathological changes such as necrosis initiation and progression as well as pathogen hyphae growth behavior revealed valuable information on genotype-specific resistance response. The individual plants were grown in a small pot (9 cm in diameter), or mini-glass box (2.5 × 7.5 × 10 cm) filled with autoclaved construction sand. For one-time microscopic observation at specific time points (typically at 48 hpi or 72 hpi occasionally),

plants were carefully excavated from pots to minimize mechanical damage to the roots. Residual soils along the root branches were gently removed under running tap water. Roots for both mock-inoculated control and *P. ultimum* inoculated plants were kept separately in a 100-mL beaker filled with autoclaved water until microscopic examination. Individual root branches were separated from each other if needed, and a glass slide was used to immobilize the root segments in a petri dish filled with water. Using this setup, a close-up image of up to 40x magnification can be captured under a dissection microscope (Olympus SXZ12). For continuous and real-time observation or timelapse imaging on the necrosis progression, a small glass box was used to hold individual plants by aligning the root system against the glass plate and separating roots from the soil substrate with a sheet of paper towel. The glass box was sealed with cotton at the top and wrapped with aluminum foil to minimize unnecessary light exposure to the root system except during observation. Grid lines on the glass plate can be used to track the specific sections of a root system between observations. Images were acquired using a DP73 digital camera installed on an Olympus SZX12 dissecting microscope with cellSense software (Olympus, Center Valley, PA). Using these in-house developed methods, the timelapse image series uncovered the contrasting features of necrosis expansion along apple roots between resistant and susceptible apple rootstock genotypes [17] [31]. At the root system level, swift development of root necrosis was commonly observed for the more susceptible genotypes, with the entire root system becoming necrotic within a period of 24 hours after an initial infection site was identified. For the more resistant genotypes, a much slower necrosis progression was observed along the infected root tissues [17]. In addition, a well-defined "zone" or "line" abruptly separating the healthy and necrotic root sections was frequently observed along the infected roots of resistant genotypes (**Figures 3(a)-(c)**), but the similar phenomenon was rare for the more susceptible genotypes [17] [30]. Also, the profuse growth of *P. ultimum* hyphae was frequently associated with infected roots of susceptible genotypes (**Figure 3(d)** and **Figure 3(e)**), but not resistant genotypes. Such stopped or interrupted necrosis progression along the roots of the resistant genotypes, such as O3R5-161, indicated an effective defense activation where the pathogen spread, and tissue collapse were substantially limited or abolished. In contrast, the swift expansion of necrotic tissues accompanied by profuse hyphae growth along the root branches of the susceptible genotypes, such as O3R5-132, clearly demonstrated an inability to suppress pathogen progression [17]. These microscopic features on infected root tissues appeared to align with whole plant resistance levels between resistant and susceptible genotypes [17] [30] [31]. These closeup observations at tissue and cellular levels provided valuable information to eventually connect specific gene and pathways with observed resistance traits in apple root to *P. ultimum* infection. Indeed, guided by the findings from parallel transcriptome analyses, such as the upregulated phenylpropanoid pathway and the induced lignin biosynthesis during apple root defense activation, more targeted studies are being carried out at cellular, subcellular and biochemical layers.

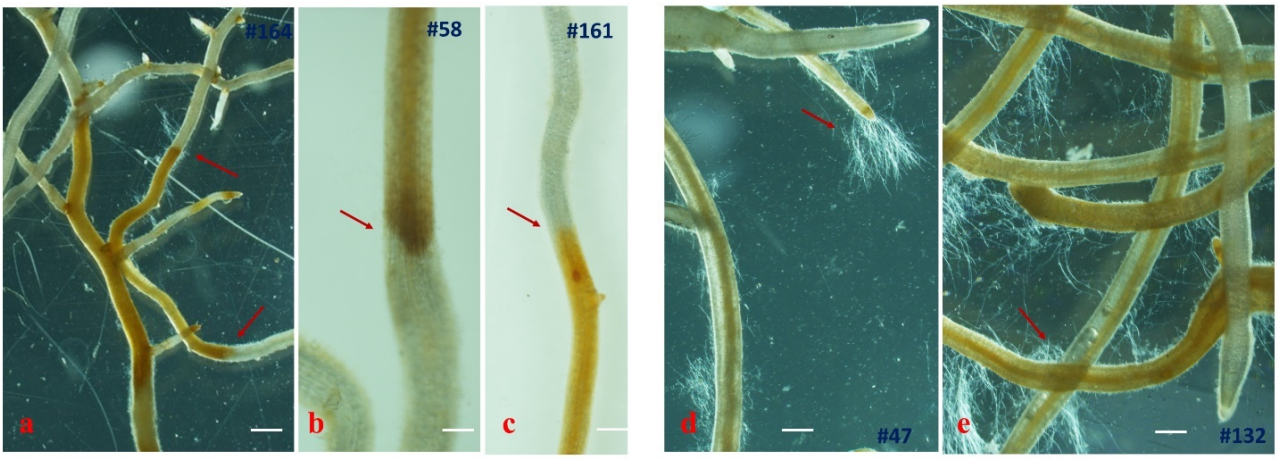

Figure 3. Microscopic features of genotype-specific resistance responses. (a)-(c), the defined lines or zones (arrows) which clearly separate healthy and necrotic section along infected root branches were observed for resistant genotypes of O3R5-#164, O3R5-#58 and O3R5-#161. (d) and (e), the wide-spread necrotic sections with typical brown coloration and profuse pathogen hyphae growth (arrows) observed in root of susceptible genotypes such as O3R5-#47 and O3R5-#132. Digital images were captured using DP73 digital camera installed on an Olympus SZX12 dissecting microscope, the obtained images were minimally modified such as re-sizing, cropping, and adjusting overall brightness of entire images using the publicly available software Faststone Image Viewer 5.5 (www.faststone.org). Bars represent 400 μm.

4.4. Cellular and Biochemical Changes Potentially Associated with Apple Root Resistance Traits

Characterization of cellular, subcellular, and biochemical changes in apple root tissues in response to *P. ultimum* infection will be essential to unlock the underline resistance mechanisms and genetic controls of observed resistance traits. Along with the effort of defining the genotype-specific resistance responses in apple roots, a series of transcriptome analyses have generated a panoramic view of transcriptional regulation over defense response in apple roots to infection from *Pythium ultimum*. Several key pathways appeared to be directly associated with apple root resistance traits. One key focus has been the potential roles of induced lignin deposition as a key factor contributing to the observed resistance traits. Lignins represent the second most abundant biopolymers after cellulose, which form covalent links with cellulose in cell walls [36] [37]. Lignin biosynthesis is the result of oxidative polymerization of three *p*-hydroxycinnamyl (*p*-coumaryl, coniferyl and sinapyl) alcohols which is catalyzed by both laccases and peroxidases [36]. The lignification process is crucial for several aspects of plant physiology including preserving the integrity of plant cell wall and imparting strength of vascular tissues [36] [38] [39] [40]. Additionally, accumulating evidence indicates that lignified cell wall serves as physical barriers against invasions of phytopathogens and other environmental stresses [41] [42] [43] [44]. Lignins are one of several groups of molecules in plant tissues that are autofluorescent, and the two most studied molecules are chlorophyll (orange/red fluorescence) and lignin (blue/green fluorescence) [45] [46]. Structural components in lignin molecules such as phenolic rings and conjugated double bonds are important organic fluorophores [45] [46]. Therefore, lignin can be detected

by histochemical staining such as Wiesner or Maule tests for brightfield observation, its autofluorescent nature also offers the advantages of label-free imaging to visualize the intensity, timing, and location of lignin deposition.

Methods for studying microscopic features of lignin deposition in young apple roots are being developed using fluorescence microscope imaging (from an Echo Revolve microscope, ECHO Discovery, San Diego, CA, USA) and histochemical staining using both Wiesner and Maule tests (Figure 4). Quantified variations of acid-soluble lignin are also being developed for measuring changes in lignin content between genotypes and in response to apple root (using Tecan, Infinite 100 microplate reader). Both brightfield and fluorescence images indicated that elevated intensity of lignin deposition can be detected in the parenchyma cells of the infected root. The genotype-specific lignin deposition around vascular bundles suggests intrinsic differences in lignin richness and/or monolignol composition between genotypes. Although lignin is not the only autofluorescent compound in cell walls, the observed quenching effect on fluorescence caused by chemical staining is consistent with the notion that lignin deposition is a primary component contributing to the detected fluorescence in young apple roots. Other pathways and genes that were identified by transcriptome analysis were also examined to validate the transcriptome data with suggested

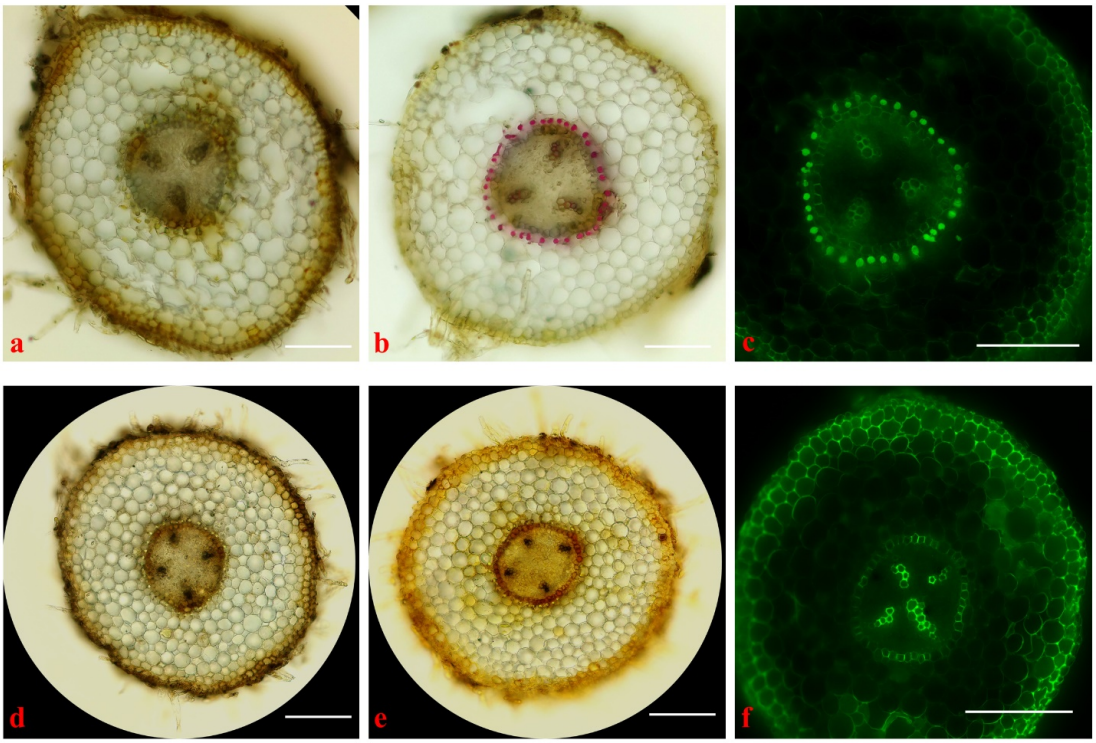

Figure 4. Histochemical staining and Fluorescence imaging for examining the microscopic features of lignin deposition in apple root. (a)-(c). Brightfield images from Wiesner test and fluorescence image for lignin deposition patterns using O3R5-#121. (d)-(f). Brightfield images from Maule test and fluorescence image for lignin deposition patterns using O3R5-#121. (d) is no-stain control, (e) is image after Maule staining procedure. (f) is the image from fluorescence microscope showing autofluorescent of lignin deposition in root tissues. Bar represents 200 μm.

direct connection with resistance trait such as genes/enzymes in phenylpropanoid pathways, and superoxide generation. From surveying population-level resistance level, and necrosis progression pattern at the tissue level, the genotype-specific variation at the cellular and subcellular levels will provide critical evidence to identify the genetic controls underlying resistance traits in apple root to *P. ultimum* infection.

5. Discussion and Perspective

High-quality phenotypic data and detailed assessment of a target trait remain the major bottleneck for identifying its molecular mechanisms and genetic controls to develop molecular tools for crop improvement [47]. Plant roots provide the vital functions of nutrient and water acquisition, anchorage, and interaction with surrounding soil environments. However, the hidden nature of roots beneath the ground significantly impedes the direct and detailed observations of their growth, and differentiation, particularly their interaction with soilborne pathogens. At the same time, root morphology and function are significantly influenced by biotic and abiotic stresses [48]. In an ideal setting, it would be great that root interaction with pathogens can be investigated using undisturbed time-lapse imaging of roots in situ under greenhouses or field conditions. Then the details of physiological or pathological processes can be documented and analyzed from collected images [48]. In reality, it is tremendously challenging to track and document root response under pathogenic pressure, especially the variation of response can be subtle between genotypes at tissue and cellular levels. The hidden nature of roots and their irregular growth patterns can complicate tissue sampling, data interpretation between treatments, genotypes, or infection events, especially for those assays focusing on cellular and biochemical responses. Investigating the resistance responses in the root tissues of apple, a Rosaceae tree fruit crop is particularly challenging [8] [9].

Pathogen infection of root tissue is ultimately a destructive process, but the reliable data of genotype-specific resistance responses come from repeated infection assays. Therefore, the continuing availability of new apple plants with defined genetic backgrounds, and consistent and comparable physiology has been a major obstacle for carrying out a systematic and detailed investigation of apple root resistance responses. By implementing the synchronized micropropagation procedure, the simultaneous availability of apple plants for multiple genotypes allowed us to identify and compare the subtle variations of their responses. Additionally, standardized inoculation protocol with a quantified inoculum level made it possible for a consistent evaluation of detailed responses between genotypes and infection events. Finally, multiple methods were tested and adapted which facilitated the reliable and in-detailed characterization of root resistance responses.

Resistance or tolerance of apple rootstock to disease pressure from soilborne pathogens was traditionally tested under field conditions [49] [50]. In those ex-

periments, the available plant materials were stool-bed propagated one-year-old rootstock "sticks" or bare-root trees from commercial nurseries [51] [52] [53]. Disease resistance or tolerance was indirectly inferred from physiological parameters at the end of the season, including tree height, stem diameter, and accumulated fruit yield. While experiments using these nursery-derived materials can provide overall rootstock performance against disease pressure under field conditions, those physiological parameters can be influenced by multiple factors including root regeneration dynamics, efficiency in nutrition uptake, adaptability to certain soil types and scion-rootstock interactions [51]. Therefore, intrinsic disease resistance responses can be masked by the interplay among many factors during field-based evaluation. Moreover, the availability of these one-year-old bareroot trees is generally restricted to a few elite commercial varieties and during a short time window. Perhaps more relevant is that the root systems of these trees have been exposed to various soil microbes or impacted by unintended abiotic conditions before standardized infection assays. Therefore, micropropagation of apple plants provides the crucial platform that made it possible this systematic analysis of several dozens of apple rootstock genotypes.

Various inoculation methods and inoculum levels were tested using two rootstock genotypes, G.935 and B.9 as examples before expanding the evaluation to O3R5 apple rootstock genotypes [30] [31]. The root-dipping method with a pathogen inoculum concentration of 2×10^3 oospores was demonstrated to be an effective inoculum dose to distinguish the resistance levels between genotypes [31]. The use of small glass boxes and microscope-assisted real-time observations uncovered detailed and closeup features of apple root resistance responses in a continuous, non-destructive fashion. Given the challenging nature of phenotyping root interactions with soilborne pathogens, these innovative methods pave the way for defining detailed resistance responses with consistency and repeatability [30] [31]. As a result, a wide spectrum of resistance responses was recorded between tested genotypes, from single digit to over 95% survival rate. Microscopic examination of root necrosis patterns provided valuable insight into the potential mechanisms underlying genotype-specific survival rates. Perhaps more importantly, the resulting plant materials, *i.e.*, apple rootstock genotypes with defined resistance responses, are the much-needed plant materials for meaningful genomics and transgenics analyses to uncover the underlying mechanisms of apple root resistance traits.

With available datasets from a series of transcriptome analyses and well-defined apple rootstock genotypes, it is feasible to investigate the molecular, cellular, and biochemical natures of apple root responses to *P. ultimum* infection. Guided by the findings from transcriptome analyses, multiple hypotheses are currently being tested. One of the most notable findings from our transcriptome data is the upregulated genes with annotated functions of phenylpropanoid and flavonoid biosynthesis, secondary metabolite transportation and laccase for lignin formation [16] [20] [54]. For example, in roots of resistant genotypes, these genes of-

ten demonstrated an early, strong, and consistent upregulation. On the contrary, in the roots of susceptible genotypes a chaotic, disrupted, and delayed expression pattern of these genes was commonly observed [16] [54]. Several laccase encoding genes were dynamically regulated by microRNAs during apple root defense against *P. ultimum* infection. These findings suggest that enhanced cell wall fortification from induced lignin biosynthesis could plays a crucial role in an effective defense activation in apple roots. Currently, several families of candidate genes from phenylpropanoid biosynthesis pathway and lignin deposition-related processes were studied for their potential association with the observed resistance traits.

Specifically for the role of induced lignin deposition and resistance, the hypothesis being tested is that there is a close connection among three elements: 1) Genotype-specific survival rate; 2) formation of the "defined zone" separating healthy and necrotic regions in the resistant genotype; and 3) elevated lignin deposition at the "defined zone". In other words, an early, quick, and strong defense activation including induced lignin biosynthesis and deposition may function as a fortified barrier to slow down the pathogen progression. Such a mechanism may offer the critical time to regenerate new root branches, which leads to increased survivability as observed for resistance genotypes. Induced lignin deposition is known to play a crucial role in response to pathogen infection, as proposed several decades ago [55] [56]. Recent molecular genomic and cellular studies on several pathosystems have demonstrated that induced lignification of the cell wall can serve as a physical barrier restricting phytopathogen intrusion [44] [57] [58] [59]. It is interesting to know that as cell wall lignification is a non-reversible process, there is a tight control over the induced lignification process including monolignol biosynthesis, polymerization and lignin deposition [41] [60] [61]. Therefore, it should not be surprising that rather subtle changes at lignin deposition could significantly impact the resistance trait to *P. ultimum* infection. Our continuing research should reveal more details of molecular and cellular actions and provide a clearer view regarding the genetic elements contributing to apple root resistance traits in response to *P. ultimum* infection.

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Authors' Contributions

YZ conceived the scope of this review manuscript and wrote most of the manuscript. ZZ participated in writing and was involved in manuscript revision.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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