Interactions between stress response, carbohydrate metabolism, and defense pathways modulate rootstock root-dependent fire blight susceptibility in apple (Malus × domestica)

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Abstract

Background

Although it is known that resistant rootstocks facilitate management of fire blight disease, incited by Erwinia amylovora (Burr.) Winslow et al., of apple scion cultivars, the role of rootstock root traits in providing systemic defense against E. amylovora is unclear. In this study, the hypothesis that rootstocks of higher root mass provide higher tolerance to fire blight infection in apples is tested. Several apple scion cultivars grafted onto a single rootstock genotype and non-grafted ‘M.7’ rootstocks of varying root mass are used to assess phenotypic and molecular relationships between root traits of rootstocks and fire blight susceptibility of apple scion cultivars.

Results

It is observed that different root traits display significant (p < 0.05) negative correlations with fire blight susceptibility. In fact, root surface area partially dictates differential levels of fire blight susceptibility of ‘M.7’ rootstocks. Furthermore, contrasting changes in gene expression patterns of diverse molecular pathways accompany observed differences in levels of root-driven fire blight susceptibility. It is noted that a singular co-expression gene network consisting of genes from defense, carbohydrate metabolism, protein kinase activity, oxidation-reduction, and stress response pathways modulates root-dependent fire blight susceptibility in apple. In particular, WRKY75 and UDP-glycotransferase are singled-out as hub genes deserving of further detailed analysis.

Conclusions

It is proposed that low root mass may incite resource-limiting conditions to activate carbohydrate metabolic pathways, which reciprocally interact with plant immune system genes to elicit differential levels of fire blight susceptibility.
Introduction

Roots play critical roles in plant function and their interactions with biotic and physical environments. Plant roots are increasingly recognized for their role in modulating systemic defenses of plants against pathogen infections via inter-organ signaling [1, 2, 3, 4]. Roots can trigger physiological and genetic responses leading to activation of molecular pathways to recognize and resist pathogens upon infection [2, 3, 4, 5]. Indeed, some root traits can act as physical barriers to soil-borne pathogens by hindering their penetration into living tissues [6, 7, 8, 9]. Therefore, investigating interactions between roots and pathogens, as well as their relationships to disease susceptibility is of particular relevance to fruit tree crops wherein specific rootstocks are frequently chosen to confer disease resistance for susceptible scion cultivars [10, 11].

It has been reported that root system architecture (RSA) is dictated by growth, length, diameter, density, branching pattern, and branching angle of various root types, and it influences resource uptake from the soil [12, 13, 14, 15]. In general, plant roots consist mainly of either one or more primary roots that, in-turn, produce several secondary and tertiary roots [15, 16]. In contrast, the root system of apple rootstocks in commercial orchards consists mainly of adventitious roots originating from nodal junctions of stem cuttings, via vegetative propagation, which are important for initial establishment and success of grafted scions. Thus, growth and density of adventitious roots can influence the nutrient acquisition capacity of a plant, both under normal and stress prone conditions [17]. Nutrient uptake not only supports overall plant growth, but it also contributes to plant survival under different stress conditions, such as wounding, flooding, drought, and nutrient deficiency [17, 18, 19]. However, the potential role of adventitious roots in enhancing tolerance to biotic and abiotic stresses remains unclear.

Rootstocks impact scion genotypes in many different ways. They can influence scion vigor
and architecture, phenology, precocity, fruit quality, and production [20, 21]. In addition, rootstocks confer differential tolerance to salinity, drought, and disease-prone conditions in various crops [21, 22, 23]. For example, resistant rootstocks have been selected and used to enhance disease tolerance of grafted scion cultivars [11, 24, 25, 26] for sustainable disease management in commercial apple orchards. It has been proposed that rootstocks can modify scion phenotypes by altering levels of abscisic acid, cytokinin, auxin, and other hormones through long-distance signaling between roots and shoots [27, 28, 29, 30]. Moreover, rootstock-regulated gene expression differences and mobile mRNA movements may also contribute towards enhanced host defense against pathogen infection [11, 25, 31]. For example, rootstocks influence expression levels of disease-associated genes of jasmonic acid and inositol pathways in grafted apple scions under fire blight infection [11]. These rootstock-derived mobile mRNAs may act as long-distance signals [31] to alter expression of disease-related molecular pathways in grafted scions. Roots also produce secondary metabolites, such as nicotine, furcoumarins, and aldehydes to improve plant defense mechanisms against pathogens [4]. In contrast, foliar bacterial infection alters secretion of malic acid in roots to recruit beneficial soil bacteria and improve plant immunity against pathogen attack [32]. Furthermore, it is likely that rootstocks may also influence scion physiology by regulating levels of water and nutrient uptake [22, 33], which in turn can impose limits on pathogen spread and disease infection. Overall, root traits of rootstocks can play critical functional roles in regulating above-ground plant physiology and disease susceptibility of scions.

Fire blight, a systemic bacterial disease incited by Erwinia amylovora (Burr.) [34] causes extensive apple production losses worldwide. Fire blight infection can occur at multiple stages of plant development with higher risks of infection occurring particularly in new growing tissues of young orchards [35, 36]. Apple growers mainly rely on use of chemical
treatments and of pruning of infected twigs to control fire blight in commercial orchards, but these preventive control measures remain inefficient once bacteria have already invaded reproductive and/or vegetative plant tissues. Plant resistance provides alternative options for sustainable control of bacterial spread, particularly once bacteria penetrate host tissues.

Use of resistant rootstocks serves to directly manage fire blight infection of rootstocks, but it can also limit its spread to susceptible scions [26, 36]. For example, susceptible scion cultivars grafted onto G.16, G.30, and G.11 apple rootstocks from the Geneva, New York apple rootstock breeding program have demonstrated high to moderate levels of resistance against fire blight [26, 37]. This observed rootstock-driven differential fire blight resistance of grafted scions is attributed to changes in gene expression of disease-related proteins and pathways, including those of phytohormones, transcription and signal transduction activities, as well as of various cellular and metabolic responses [11].

Rootstocks can potentially utilize several mechanisms to confer resistance to scions, but the precise mechanism of rootstock-defined scion resistance or tolerance to fire blight remains unknown.

In this study, we have tested the hypothesis that apple rootstocks of higher root mass (g) can respond more effectively to fire blight infection. To pursue this, following two experiments have been conducted. In one experiment, a range of apple scion cultivars are grafted onto a single apple rootstock, ‘Malling 7’ (‘M.7’), grafted trees are allowed to grow, and are then challenged with artificial inoculation with *E. amylovora*. In the second experiment, non-grafted ‘M.7’ rootstocks of varying root mass are grown, and then these are challenged with *E. amylovora*. Morphological characteristics were evaluated for both the experiments that lead to assessment of molecular interactions between root traits of rootstock and fire blight susceptibility of apples.
Results

Rootstock Root Traits and Fire blight Susceptibility of Grafted Scions are Correlated

Data from 45 grafted scions on M.7 rootstocks were used to evaluate the relationships between root traits and fire blight infection traits from shoot and leaf. Both root dry mass (g) and average roots per node (count) showed a wide range of variation in this population of scion genotypes. The root dry mass of M.7 rootstocks varied from 0.67 grams (g) to 6.84 g (Fig. 1A), whereas average number of roots per node ranged from 2.96 to 7.56 in M.7 rootstocks (Supplementary Fig. S1). Furthermore, corresponding shoot and leaf traits also showed significant \((p < 0.05)\) variations in this population (Supplementary Fig. S1). Specifically, shoot and leaf lengths varied from 8.8 to 24.5 cm and 3.0 to 6.6 cm in this population, respectively, while SPAD values for leaf chlorophyll measurements ranged from 28.4 to 38.5. Moreover, shoot traits showed moderate broad-sense heritability \((H^2)\) values, ranging from 0.70 to 0.62, for leaf length and for leaf chlorophyll content. Of particular interest, percent lesion length ranged from 1.2% to 93% and showed significant \((p < 0.05)\) variation in the population (Fig. 1B). The \(H^2\) values for percent lesion length was 0.71.

Pairwise phenotypic correlations showed positive correlations between root and shoot traits, and negative correlations between root and fire blight susceptibility traits (Supplementary Table S1). However, not all correlations were significant. For instance, root dry mass (g) had significant \((p < 0.05)\) negative correlation of -0.45 with percent lesion length (Supporting Fig. S2), whereas negative correlations between average roots per nodes and fire blight susceptibility traits were not significant. Similarly, root dry mass (g) displayed significant \((p < 0.05)\) positive correlations with leaf length, but not with
shoot length (Supplementary Table S1). Overall, these phenotypic correlations suggested that root dry mass of the rootstock could influence leaf growth and fire blight susceptibility of grafted scions.

Hierarchical clustering and multivariate analysis were used to categorize the entire population into groups based on their phenotypic differences. Therefore, this population was divided into six main clusters exhibiting distinct trait variation patterns, as illustrated in the heat map of traits values (Supplementary Fig. S3). Furthermore, PCA of root and fire blight disease traits also highlighted phenotypic differences of these genotype clusters in this population (Fig. 2A). For example, root dry mass (g) and percent lesion length (%) showed variable distribution patterns of trait means among the six identified clusters (Fig. 2B and 2C). In fact, clusters “C4” and “C6” tended to consist of genotypes with relatively lower root mass (g) and higher disease susceptibility. However, this observed pattern was less clearly demonstrated in the remaining clusters.

Interestingly, nine principal components (PCs) explained the total variation present in this population. PC1 explained a maximum of 32.6% of the total variation. Although all traits contributed towards PC1 variation, root and shoot traits had positive contributions, while fire blight disease susceptibility traits contributed negatively to PC1 variation (Supplementary Fig. S4). This trend supported previously detected negative correlations between root and fire blight susceptibility traits. In addition, analysis of higher-order PCs revealed different levels of contributions from root, shoot, and disease susceptibility traits (Supplementary Fig. S4), as noted by positive contributions from all root and disease-related traits to the PC2 variation. Overall, the PCA analysis revealed presence of considerable variation in this population, and this was partially driven by identified correlations between root growth and disease susceptibility traits.

Rootstocks Exceeding a Root Area Threshold are less Susceptible to Fire
Blight

To evaluate the extent to which roots can influence levels of fire blight susceptibility, a second independent experiment was conducted using non-grafted M.7 rootstocks representing four distinct root area classes (RACs). It was found that average root surface areas ranged from approximately 1,720 (lowest RAC-1) to 4,455 cm² (highest RAC-4), corresponding to about 1.27 to 2.59-fold change between the lowest and the other three RACs (Fig. 3A). Moreover, fire blight infection, measured as percent lesion length (%), showed significant (p < 0.05) variations among the different RACs over time (Supplementary Fig. S5). It was observed that absolute rates of disease progression from 2 to 8 dai were about 41% to 75% in RAC-4 and RAC-3; whereas, these were higher, 84-98%, in RAC-1 and RAC-2 (Supplementary Fig. S5). At 8 dai, percent lesion length was significantly (p < 0.05) different in RAC-1 from those of RAC-3 and RAC-4, while this was not significantly different, at p < 0.05, in RAC-2 from those of the other RACs (Fig. 3B).

Overall, total infection and progression of disease were comparatively less in root classes of high root surface areas (cm²) at the start of the experiment, and vice-versa. Moreover, the highest fire blight susceptibility was observed in rootstocks with a threshold of root surface area of 3,644 cm², represented by RAC-3 (Fig. 3).

Analysis of phenotypic correlations showed a strong correlation (r² = 0.87; p < 0.05) of root areas (cm²) between pre- and post-planting (and following bacterial inoculation), for a total duration of 106 days of growth, thus indicating that initial root area could serve as a predictor of root area growth at later stages of root development. Similarly, other root traits also demonstrated significant (p < 0.05) positive correlations (Supplementary Table S2). For instance, root dry mass (g) showed high positive correlations with pre- and post-plant root area (r² = 0.82 and 0.93, respectively). Similarly, both coarse and fine root
mass showed significantly ($p < 0.05$) high positive correlations with root area (cm$^2$) before ($r^2 = 0.79$ and $0.62$, respectively) and after planting ($r^2 = 0.85$ and $0.78$, respectively). Some root traits also displayed significant ($p < 0.05$) negative correlations with fire blight susceptibility traits. For instance, pre-plant root area (cm$^2$) and fine root dry mass (g) had significant negative correlations of -0.70 and -0.58 with percent fire blight lesion length, respectively (Supplementary Table S2). In contrast, negative correlations of percent lesion length against post-planting root area (cm$^2$), coarse root dry mass (g), and total dry mass (g) were not significant.

**Contrasting expression patterns of distinct sets of genes are associated with root-dependent fire blight susceptibility**

Following bacterial inoculation, phenotypic analysis identified significant ($p < 0.05$) differences in fire blight infection over time in leaves of M.7 rootstocks belonging to different RACs (Fig. 3; Supplementary Fig. S5). To identify molecular changes related to root-regulated fire blight susceptibility, gene expression patterns were characterized in leaf tissues of contrasting root area classes (RAC-1 with an average root area of 1,720 cm$^2$ vs. RAC-4 with an average root area of 4,455 cm$^2$) of non-grafted M.7 genotypes under control and bacterial inoculation treatments, at 4 and 8 dai. This gene expression analysis was conducted in sequential steps (Fig. 4). We first compared control leaf samples between RAC-1 and RAC-4 to identify any differentially expressed genes (DEGs) accounting for effects of root surface area differences on leaf responses. Next, control and bacterial-inoculated leaf samples were analyzed over time to identify fire blight responsive genes in leaf tissues. As a result, a set of common genes from both analyses were identified and deemed as genes associated with root-regulated fire blight susceptibility responses in leaf tissues.
Subsequently, it was observed that a high PC1 variation (82%) was present in this population (Supplementary File S1), thus suggesting that root area, fire blight infection, and sampling time contributed to the variability detected in the gene expression dataset. Furthermore, the number of significant \((p < 0.05)\) DEGs increased over time in both control and bacterial-inoculated RAC-4 samples, while these decreased in bacterial-inoculated RAC-1 samples over time (Fig. 4A). Interestingly, a total of 132 and 1,017 DEGs were detected between RAC-1 and RAC-4 at 4 and 8 dpi, respectively (Table 1; Supplementary File S2). As all 132 DE genes at 4 dpi were also identified at 8 dpi (Supplementary Fig. S6), which indicated that the effects of root surface area differences persisted, and in fact they became more severe over this developmental period. In addition, a set of 454 out of 1,017 DEGs showed significant \((p < 0.05)\) changes in transcript levels following bacterial infection (Fig. 4B; Supplementary File S3). These DEGs involved genes likely related to bacterial infection, whose expression levels were also dependent on differences in root surface areas, as noted in contrasting RACs, and referred to herein as root-regulated fire blight responsive (RRFBR) genes. An analysis of normalized expression for RRFBR genes identified opposite trends in contrasting RACs at both 4 and 8 dai. For instance, about 31.7\% \((n = 144)\) DEGs demonstrated increased expression levels in RAC-1, but decreased expression levels in RAC-4 following bacterial inoculation at both sampling times (Supplementary File S4). Likewise, 11.4 \% \((n = 53)\) DEGs demonstrated decreased expression levels in RAC-1, but increased expression levels in RAC-4. Interestingly, only a few genes \((n = 9)\) demonstrated similar patterns of changes in expression levels between two these RACs following bacterial infection (Supplementary File S4). These findings suggested that for the majority of RRFBR genes, differences in fire blight susceptibility between RAC-1 and RAC-4 were mostly associated with contrasting gene expression patterns.
Interactions between genes from multiple pathways accompany root-dependent fire blight susceptibility

Upon further gene ontology (GO) analysis, it was noted that ~92% of RRFBR genes belonged to general stress response pathways related to metabolic response (42%), catalytic activity (40%), and oxidation-reduction (10%) processes (Fig. 4C; Supplementary File S5), whereas 8% of RRFBR genes represented functional terms related to carbohydrate metabolic process, cell recognition, response to biotic stimulus, protein serine/threonine kinase activity, and apoplast (Fig. 4C; Supplementary File S5). Moreover, expression levels of DEGs varied within these pathways (Supplementary Fig. S7). For example, some DEGs in carbohydrate metabolic and protein kinase pathways had lower levels of expression in RAC-1 than in RAC-4, while other DEGs displayed an opposite trend (Supplementary Fig. S7). Interestingly, all six DEGs involved in defense response pathways demonstrated reduced levels of expression in RAC-1, but increased levels of expression in RAC-4 at 8 dai following bacterial infection (Fig. S7; Supplementary File S5). Overall, these findings pointed toward likely interactions of both general stress response and carbohydrate metabolism pathways with defense-related genes. Indeed, these interactions would explain root-regulated differences in fire blight susceptibility in apple.

Subsequently, weighted co-expression analysis was used to identify co-expression patterns and putative interactions among RRFBR genes by identifying those hubs with the highest intramodular connectivity (Fig. 5A). It was found that a singular co-expression module “C3” represented 77.3% of RRFBR genes (Supplementary Fig. S8). In addition, UDP-glycosyltransferase, formate dehydrogenase, pathogenesis-related 4, WRKY DNA-binding protein 75, cysteine-rich RLK, cytochrome P450, laccase 7, glucose-methanol-choline (GMC) oxidoreductase protein, and glycosyltransferase family proteins were found
to be consistently present as highly connected genes in the “C3” module (Fig. 5B; Supplementary File S6). This suggested that interactions among core genes from the general stress response, carbohydrate metabolism, and defense pathways determined observed differences in fire blight susceptibility between RAC-1 and RAC-4. In addition, detection of DNA-binding domain proteins as hubs in the “C3” module supported transcriptional regulation of co-expressed genes from these different pathways.

Discussion

Earlier studies have reported that rootstocks and rootstock system architecture influence various important traits of scion genotypes grafted onto these rootstocks [20, 21]. As it has been demonstrated that rootstocks confer enhanced tolerance to salinity, drought, and disease in various crops [21, 22, 23], efforts have been undertaken to develop resistant rootstocks, which in turn can enhance disease tolerance of grafted scion cultivars [11, 24, 25]. In this study, it is observed that root traits of an apple rootstock (M.7), including root dry mass (g) and average roots per node (count), are indeed variable, and they do in turn influence shoot and leaf traits, including leaf chlorophyll contents, of different scion genotypes grafted onto this rootstock. More importantly, these root traits also influence response reactions of leaf and shoots of different scion genotypes to controlled inoculations with *E. amylovora*, and their susceptibility to fire blight disease. Although this latter finding confirms earlier reports [24, 26], it provides detailed analysis of the importance of root mass traits on fire blight reactions of grafted scion genotypes upon infection by *E. amylovora*. This finding is further supported by significant (*p < 0.05*) negative correlations obtained between root mass and scion fire blight susceptibility in these grafted apple trees.

Upon analysis of RACs of non-grafted ‘M.7’ rootstocks on fire blight susceptibility of above-ground leaf tissues, it is observed that increased root surface area contributed to
decreased fire blight susceptibility in these above-ground leaf tissues, and the reverse is found to be true as well. These observed root-dependent differences in levels of fire blight disease susceptibility may be attributed to presence of multiple system level defense mechanisms [11, 25, 38, 39, 40]. Indeed, involvement of diverse molecular pathways related to plant metabolism, cell cycle, oxidation-reduction, and stress response suggests presence of systemic regulation of fire blight infection in apples [11, 38]. Moreover, rootstock genotypes can significantly contribute to scion tolerance to fire blight susceptibility via rootstock-regulated gene expression patterns [11].

It is important to note that different fire blight disease reactions and root traits have displayed significant (p < 0.05) levels of variations in the two experiments conducted in this study. The observed variation in percent lesion length (%) could be attributed, in part, to the different genetic backgrounds of grafted scion cultivars, which was supported by calculated moderate broad-sense heritabilities of percent lesion length (%). Although grafted scions could also influence root traits of M.7 rootstock, which is less likely to have occurred in this study, as each experiment was conducted within a short time frame (~2 months). It has been reported that variations in root traits of rootstocks might contribute to phenotypic plasticity due to their exposure to different nutrient regimes, soil, and environmental conditions during their earlier growth in the clonal rootstock [15, 41, 42]. Phenotypic plasticity of different traits can vary among different genotypes [43, 44, 45, 46]. Thus, identifying genes for root phenotypic plasticity would support efforts to breed for rootstocks with more uniform root traits. This, in turn, would contribute to enhanced resistance against *E. amylovora* infection, particularly for young grafted apple trees grown in orchards.

In this study, detection of various DEGs, including several disease-related and pathogenesis proteins, between contrasting RACs point to the critical role of the central
immune system in conferring root-dependent fire blight susceptibility/resistance reactions. Earlier studies have reported that some of the disease-related CC-NBS-LRR proteins confer major resistance against fire blight in apples [39, 40]. These results suggest that gene interactions between core defense pathways and system-level metabolic and stress-responsive pathways may regulate root-dependent fire blight susceptibility/resistance reactions in apple. Moreover, these pathways may operate in coordination with sugar and carbohydrate metabolic pathways, which have demonstrated overrepresentation in contrasting RACs investigated in this study. Thus, it is likely that low root mass alters sink activities of a plant, which in turn can modify expression patterns of carbohydrate metabolism genes. Furthermore, changes in carbohydrate metabolism can alter a plant's defense response through an inter-connected signaling network of metabolic and stress-responsive genes. For example, restriction of below-ground root growth can alter both development and carbohydrate metabolism of above-ground leaf tissues [47, 48, 49]. In addition, alteration in metabolite levels in a source leaf can determine the defense response against pathogen infection [50, 51 52]. In fact, detection of a single co-expression module, “C3”, consisting of carbohydrate metabolism and disease-related proteins further supports viability of such a model in apple. Furthermore, presence of interacting WRKY and ethylene responsive DNA-binding transcription factors suggest transcriptional co-regulation of these pathways. Thus, low root mass may lead to resource-limiting conditions in the plant, thereby contributing to changes in gene expression of pathogenesis and disease-related proteins through carbohydrate metabolism pathways. It is these changes in expression in the central plant immune system that would then eventually determine fire blight susceptibility levels in apple.

It is important to point out that co-expression analysis conducted in this study has also highlighted those core genes with the highest intra-modular connectivity. In particular, the
WRKY75 transcription factor and an UDP-glycotransferase are the top two genes displaying highest levels of connectivity within this network. Thus, these two genes are deemed as worthy candidates for further studies to assess their potential roles in various biotic and abiotic stress conditions. Furthermore, it will be interesting to identify those factors contributing to root-dependent differences in levels of fire blight susceptibility/resistance. The root system directly regulates amounts of nutrients and water uptake, which in turn influence growth, physiology, and metabolism of grafted scions [22, 33, 53]. As smaller root systems can limit availability of nutrients or impose partial stress conditions, this in turn can influence levels of disease susceptibility. For instance, water deficit is reported to increase plant susceptibility against fungal infections in different plant species [54, 55]. This is partially attributed to altered expression of host R genes and/or of pathogen effectors [54]. In this study, contrasting expression patterns of several disease resistance genes and leucine rich repeats have been detected between RAC-1 and RAC-4, thus suggesting incidence of changes in plant immunity under low root surface areas. Similarly, nitrogen availability can affect disease severity levels in plants [53, 56]; however, the precise mechanism of nutrient-dependent changes in disease susceptibility levels remains unknown. Therefore, further studies should be conducted to determine the role(s) of resource limiting conditions and those factors involved in differences in root-dependent responses to fire blight susceptibility/resistance reactions in above-ground plant tissues.

Conclusions

In summary, roots of a rootstock can influence levels of fire blight susceptibility of apples. An optimum root area threshold is required to achieve the maximum tolerance against fire blight; however, high plasticity of root traits can hinder maintenance of such an optimal root system in apple rootstocks. Therefore, further studies should be conducted to identify genes or growth conditions that control root size, branching, and root phenotypic
plasticity, as this new knowledge will assist in efforts to design more uniform root systems for optimum vigor of clonal apple rootstocks. In addition, manipulation of core regulatory genes of stress-responsive pathways can contribute to enhanced plant tolerance to abiotic and biotic stresses imposed by restricted root growth and disease infection.

Materials And Methods

Plant Material and Growth Conditions

One-year-old apple rootstocks of ‘Malling 7’ (‘M7’), a moderately fire blight-susceptible rootstock, were purchased from Willamette Nurseries Inc. (Canby, OR), and used in two different experiments. ‘M.7,’ a commercially important apple rootstock, was originally selected from traditional French rootstocks, known as ‘Doucin’, at East Malling Research Station (UK).

To evaluate influence of root mass of rootstocks on fire blight susceptibility of grafted scions, 45 different scion genotypes were grafted onto 1-year-old ‘M.7’ rootstocks. These grafted trees were maintained in a moist dark chamber for a period of three months to promote healing of the graft unions. Then, these grafted trees were planted in D40H deepots (Stuewe and Sons, Tangent, OR), 6.5 cm in diameter and 24.2 cm in depth, containing a standard Cornell soil mix (50 peatmoss:50 vermiculite with 6.2 kg.m⁻³ lime, 1.25 kg.m⁻³ superphosphate, and 0.62 kg.m⁻³ calcium nitrate). These trees were allowed to acclimatize and grow in a greenhouse facility at Cornell AgriTech (Geneva, NY) maintained at 25°C, 50% RH, and 16 h light/8 h dark photoperiod for a period of 8 weeks. For each scion genotype, three replications were maintained in the greenhouse facility at the Cornell AgriTech (Geneva, NY), and arranged in a completely randomized block design. To assess the effects of varying root mass (g) on fire blight susceptibility, 21 non-grafted ‘M.7’ rootstocks were used in a second experiment. One-year-1-year-old ‘M.7’ rootstocks
were pruned from the bottom up, using Fiskars hedge shears, to alter the numbers of adventitious root nodes growing along each of the rootstocks. The resulting rootstocks were photo-imaged, and analyzed using ImageJ (https://imagej.nih.gov/ij/) to identify four different root area classes (RACs). These non-grafted ‘M.7’ rootstocks were then potted in plastic pots (26 cm in diameter and 22.5 cm in depth) using the Cornell Soil mix as described above. For each RAC rootstock treatment, three replications were used, and these trees were maintained in the greenhouse facility at Cornell AgriTech, arranged in a completely randomized block design, under conditions of 25°C, 50% RH, and 16 h light/8 h dark photoperiod for a period of 106 days.

Fire Blight Inoculation and Trait Evaluation

Potted young trees were inoculated with either bacteria (treatment) or water (control) for fire blight evaluation. Bacterial inoculum was prepared using a highly virulent *E. amylovora* strain, Ea2002A. Frozen inoculum stock was transferred to a petri plate containing King’s B medium (KB), and incubated for 48 h at 28°C. Bacterial cells were recovered in a suspension culture using 1X PBS, and adjusted to a concentration of $10^9$ CFU/ml on a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The youngest unfolded leaf of an actively growing shoot of a potted young tree was inoculated by bisecting across the midribs using scissors dipped in the bacterial suspension, as described earlier [38, 57]. Deionized water was used to bisect midribs of leaves of control plants.

All inoculated young trees were evaluated 8 days after inoculation (dai) for fire blight infection, and for root traits. For both experiments, total shoot length, total leaf length, and length of necrosis of a leaf were measured in ‘cm’ using a ruler. The percent leaf lesion length (%) was calculated as the ratio of necrotic lesion length of a leaf to total leaf
length multiplied by 100. Furthermore, chlorophyll contents of control and infected leaves were measured using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, Aurora, IL, USA). Average roots per node (count) and root dry mass (g) were simultaneously evaluated for all inoculated young trees (control and fire blight treated). Average roots per node were calculated by dividing total number of roots by number of nodes of a rootstock cutting used. At the end of each experiment, roots were shaved off each of the rootstocks, and dried in an oven to determine dry root mass. For the second experiment, additional root trait data were digitally collected both at the beginning and at the end of the experiment. The root system from each ‘M.7’ rootstock was photographed by rotating it 360 degrees to capture the three-dimensional root surface area using a Canon EOS Rebel T5 Digital SLR camera (Canon USA Inc., Melville, NY, USA). All raw images were first converted into greyscales, and then followed by binary conversion using the software ImageJ (https://imagej.nih.gov/ij/). Binary images were used to calculate the total root surface area (cm$^2$) at the beginning of the experiment. Rootstocks were categorized into four different RACs, from lowest to highest root surface area (cm$^2$). At the end of the experiment, roots were carefully dug out, and washed using a detergent and water. Roots were then spread on a flat surface, and photographed using a Canon EOS Rebel T5 Digital SLR camera. Photo-images were processed using an ImageJ software to calculate pre- and post-experiment root surface areas (cm$^2$). Based on digital root diameter classifications, the root system of each young tree was separated into coarse (diameter > 1 mm) and fine (diameter < 1 mm) roots, dried in an oven, and then used to determine fine root dry mass (g), coarse root dry mass (g), and total root dry mass (g).

Statistical Analysis
All data collected for root and shoot traits, as well as for fire blight disease severity were subjected to analysis of variance (ANOVA) using an R statistical software (http://www.R-project.org/). Mean values were compared using Tukey’s multiple comparison test. Broad-sense heritability (H²) was estimated as ratio of $V_G/V_P$, where $V_P$ corresponded to the total phenotypic variance explained by the genetic component variance ($V_G$).

Average trait values were used to calculate Pearson correlation coefficients, as well as to perform hierarchical clustering with the “hclust” function and a principal component analysis (PCA) using “prcomp” function in R (http://www.R-project.org/). Hierarchical clustering estimated individual relationships based on extent of similarities between them; whereas, PCA utilized variance components to determine such relationships. Trait mean values were scaled to conduct both hierarchical clustering and PCA analysis. For hierarchical clustering, scaled trait datasets were used to generate an Euclidean distance matrix for estimation of inter-cluster distance with Ward’s linkage method. PCA analysis was conducted to obtain principal component (PC) eigenvalues and rotations to estimate contributions of different traits to explain variation by each PC. A PCA biplot was generated using the first two principal components (PC1 and PC2) to determine the overall genotypic variation and effects of root dry mass (g) on disease severity.

**Leaf Sample Harvesting, RNA Extraction, 3’RNAseq Assay and Sequencing**

Leaf tissues from M.7 rootstocks of contrasting initial root surface areas (cm²), in the second experiment, were used for RNA extraction and for gene expression analysis. Leaves were collected at 4 and 8 dai from both control and bacterial-inoculated young trees of two contrasting RACs. Leaf tissues were immediately immersed in liquid nitrogen, and stored at -80°C until used for RNA extraction.

A SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to extract
total RNA as per manufacturer’s protocol. Leaf tissues were ground into fine powder in liquid nitrogen, and 100 mg of leaf powder was transferred to 500 µl of lysis solution containing 2% -mercaptaethanol. Samples were thoroughly mixed, placed at 56°C for 5 min, and centrifuged for 1 min at 13,000 rpm. The supernatant was passed through a filtration column at 13,000 rpm for 1 min to remove debris. The cleared lysate was mixed with 250 µl binding solution, and centrifuged through a binding column for 1 min at 13,000 rpm. After RNA binding, samples were washed twice using 500 µl wash solution I, as per manufacturer’s recommendations. Columns were centrifuged at maximum speed for 1 min during various washing steps. Dry columns were transferred to a new 2 ml centrifuge tube, and 50 µl elution buffer was added into the center of each binding column. Samples were kept in an elution buffer for 1 min, centrifuged at a maximum speed for 30 sec to elute RNA, and then this was repeated using 30 µl of elution buffer to increase RNA yield. The amount of RNA was determined using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Grand Island, NY, USA), and the quality of RNA samples was assessed by running a 1% bleach agarose gel.

Library construction and sequencing for RNA samples from control and bacterial-inoculated samples were performed at the Genomics Facility at Cornell University (Ithaca, NY, USA). Briefly, 3’RNAseq libraries were prepared from ~500 ng of total RNA per sample using the Lexogen QuantSeq 3’ mRNA-Seq Library Prep Kit FWD for Illumina (https://www.lexogen.com/quantseq-3mrna-sequencing/). Libraries were quantified on a Molecular Devices Spectra Max M2 plate reader (with the intercalating dye QuantiFluor), and pooled accordingly for maximum evenness. The pooled sample was quantified by digital PCR, and sequenced along a single lane of an Illumina NextSeq500 sequencer to obtain single-end 1x86 bp sequences. Pooled libraries were de-multiplexed based upon six-base i7 indices using an Illumina bcl2fastq2 software (version 2.17; Illumina, Inc., San
Sequencing Data Processing and Analysis

A Trimmomatic (version 0.36) [58] software was used to remove Illumina adapters from de-multiplexed fastq sequences, as well as to remove low-quality reads for further analysis. Poly-A tails and poly-G stretches of at least 10 bases in length were then removed using the BBDuk program in the package BBMap (https://sourceforge.net/projects/bbmap/), but keeping reads of at least 18 bases in length after trimming. Often, poly-G stretches are obtained from sequencing past ends of short fragments (G = no signal).

Trimmed reads were then aligned to the GDDH13 Version 1.1 apple genome assembly (https://iris.angers.inra.fr/gddh13/downloads/GDDH13_1-1_formatted.fasta.bz2) using the STAR aligner (version 2.5.3a) [59]. For the STAR indexing step, the gff3 annotation file (https://iris.angers.inra.fr/gddh13/downloads/gene_models_20170612.gff3.bz2) was converted into a gtf format the gffread program from cufflinks (version 2.2.1) [60]. Key parameters used in the STAR indexing step (--runMode genomeGenerate) include --genomeChrBinNbits 18 and --sjdbOverhang 100. The STAR alignment step used the following key parameters: --outReadsUnmapped Fastx, --outFilterMultimapNmax 10, --outFilterMismatchNoverLmax 0.06, --outSAMmode Full, --outSAMattributes Standard, --outFilterIntronMotifs, and RemoveNoncanonicalUnannotated. Output SAM files were converted to BAM using SAMtools (version 1.6) [61], and numbers of reads overlapping each gene in the gff3 file along the forward strand were counted using a HTSeq-count (version 0.6.1) [62]. A gene was deemed to be expressed using a criterion of detecting a minimum of five aligned high-quality read sequences against a particular gene model.
Gene Expression and Enrichment Analysis

The R package DESeq2 (version 1.20.0) [63] was used to obtain normalized counts from raw read counts. These counts were then used to conduct PCA of the 500 most variably-expressed genes following count normalization and variance stabilizing transformation, as well as for differential gene expression analysis. Control and bacterial-inoculated samples of each root class and time points were compared by deeming root class, time point, and inoculation treatment as distinct factors. The “contrast” function in DESeq2 was used to obtain expression analysis output for each comparison. For each gene, statistical significance of differential expression was based on a Wald test for a non-zero log fold change (LFC) estimate obtained from fitting a negative binominal generalized linear model [63]. Adjustment of p-values for multiple testing followed the Benjamini and Hochberg method [64]. Genes were deemed differentially expressed based on a log2Fold change threshold of 1.5 and a p-value of less than 0.01. All upregulated genes were determined based on positive log2Fold change values, and vice-versa.

Sets of differentially expressed genes from individual comparisons were used to perform a gene ontology (GO) term enrichment analysis using Fisher’s exact test with agriGO v2.0 [65]. Differentially expressed (DE) genes were compared to the complete set of fully-annotated genes in the GDDH13 Version 1.1 apple genome assembly. Estimated p-values were corrected using the Hochberg false discovery rate (FDR) correction method in agriGO v2.0. A p-value cutoff of less than 0.05 was used to determine significantly enriched GO terms.

Gene Co-expression Network Analysis

A co-expression network analysis of DE genes was performed using the weighted co-expression network analysis (WGCNA) package in R [66] to obtain modules of genes
having similar expression patterns. A list of unique DE genes from each comparison was established by removing redundant genes from various differential gene expression analyses. Subsequently, normalized gene expression values for these unique DEGs were extracted to perform a co-expression network analysis. Co-expressed gene modules were then built using a single-step network generation and a module detection approach in WGCNA. Specifically, a network was generated by connecting all expression values in a dataset followed by detection of modules exhibiting very similar patterns of gene expression. A threshold value for module assignments was estimated using an unsigned topological overlap matrix (TOM); whereas, networks were constructed using a threshold power of 16, branch cut height of 0.25, and a minimum module size of 30. These co-expression modules were then visualized in Cytoscape v3.7.1 [67], and those highly connected genes, within each module, were identified using cytoHubba plugin [68] in Cytoscape. These modules were exported from WGCNA using a threshold of 0.2, and the top 20 highly connected genes were identified using four different algorithms including MCC, MNC, Degree, and EPC, implemented in cytoHubba. Finally, outputs were compared to select those hub genes that were consistently detected from all the four algorithms.

Declarations

Availability of Data

All read sequences from RNA-Seq analysis for all samples used in this study have been deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) database under the Bioproject identifier PRJNA507638. The data supporting the conclusions of this research is provided as supplementary information.

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Conflict of interests

The authors declare that they have no competing interests.

Author’s Contributions

A.K. designed the experiment. Elsa Desnoues (E.D.) and Jack Fabrizio (J. F.) conducted the first and second experiments respectively. Julliany Silva (J.K.S.) supported in the design and data collection of second experiment. Jugpreet Singh (J.S.) performed the data analysis including gene expression, pathway enrichment and co-expression analysis. J.S. and A.K. interpreted the data and wrote the paper. Wolfgang Busch supported the conceptualization and design of the experiments, interpretation of results and reviewing of the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

There are no ethical guidelines for research on bacterial Erwinia amylovora.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Table 1

Table 1. Total number and percentage of differentially expressed (DE) genes for each comparison between two root area classes (RACs); lowest (RAC-1) and highest (RAC-4), and between control and fire blight (FB) infected samples at two time points after infection. The percentage of DE genes was calculated by comparing against total expressed genes (n=35224) in the transcriptome dataset. The genes were defined as DE based on the p-value < 0.01 and log2Fold change of 1.5 from likelihood ratio test statistics using DESeq2. In the RAC-1 vs RAC-4 comparisons, the induced genes have comparatively higher gene expression in RAC-1, whereas repressed genes have comparatively higher gene expression in RAC-4.

| Treatment               | Time | Comparison                  | No. of DE Genes | DE Genes (%) | Induced | Repressed |
|-------------------------|------|-----------------------------|-----------------|--------------|---------|-----------|
| Root Area Classes       |      |                             |                 |              |         |           |
|                         | 1    | RAC-1 vs RAC-4              | 132             | 0.37         | 24      | 108       |
|                         | 2    | RAC-1 vs RAC-4              | 1017            | 2.88         | 412     | 605       |
| Fire Blight Infection   |      |                             |                 |              |         |           |
|                         | 1    | RAC-1 (Control vs FB Treatment) | 405             | 1.15         | 377     | 28        |
|                         | 1    | RAC-4 (Control vs FB Treatment) | 52              | 0.15         | 39      | 13        |
|                         | 2    | RAC-1 (Control vs FB Treatment) | 341             | 0.97         | 240     | 101       |
|                         | 2    | RAC-4 (Control vs FB Treatment) | 980             | 2.78         | 549     | 431       |

Supplemental File Details

Supplementary Figure S1. Boxplots showing variation in different shoot traits in 45 grafted scion genotypes on M.7 rootstocks.
**Supplementary Figure S2.** Pearson correlation coefficients ($R^2$) of root dry mass (g) against percent lesion length (%) of 45 grafted scion genotypes on M.7 rootstocks.

**Supplementary Figure S3.** Hierarchical genotype clustering of 45 grafted scion genotypes on M.7 rootstocks and cluster mean heatmap for different traits.

**Supplementary Figure S4.** Barplot showing rotations of first three principal components for different traits.

**Supplementary Figure S5.** Rate of disease progression (percent lesion length) over time.

**Supplementary Figure S6.** Volcano plots showing differentially expressed genes (DEGs) at 4 dai (A), and 8 dai (B). The unique and common DEGs are also shown in Venn Diagram (C).

**Supplementary Figure S7.** Heat map showing expression patterns of DEGs in protein kinase, carbohydrate metabolism, and defense pathways.

**Supplementary Figure S8.** Heat map showing expression patterns in three co-expression modules detected from weighted gene co-expression analysis.

**Supplementary Table S1.** Pairwise correlation coefficients between root, shoot, and disease traits in 45 grafted scions on M.7 rootstocks.
**Supplementary Table S2.** Pairwise correlation coefficients between root, shoot, and disease traits in different root area classes (RACs).

**Supplementary Table S3.** Summary of sequencing and read alignments against the apple genome assembly.

**Supplementary Table S4.** Number of expressed genes at alignment criteria of 5 reads mapped to a particular gene model against the apple genome assembly.

**Supplementary File S1.** Principal component analysis (PCA) using normalized read counts of apple genes.

**Supplementary File S2.** DEGs between RAC-1 and RAC-4 from control samples at 4 and 8 dai.

**Supplementary File S3.** DEGs between control and fire blight infected samples in RAC-1 and RAC-4 at 4 and 8 dai.

**Supplementary File S4.** Functional annotations and expression patterns of common 454 DEGs.

**Supplementary File S5.** Pathways showing enrichment in the common 454 DEGs.

**Supplementary File S6.** Hub genes in the “C3” co-expression module.
Figures
Distribution of mean trait values in 45 grafted scion genotypes on M.7 rootstocks. (A) root dry mass, gram, (B) percent lesion length (%). Percent lesion length was calculated as a ratio of leaf necrosis length to total leaf length multiplied by 100.

Error bars correspond to standard deviations of means.
Figure 2

Genotype clustering based on principal component analysis (PCA) and hierarchical clustering of root and disease traits. (A) Six genotype clusters obtained from a population of 45 scion genotypes grafted onto M7 rootstocks. Distribution of means and variances in each cluster for (B) root dry mass (g) for C1- 2.3, C2- 2.2, C3- 3.0, C4- 3.8, C5- 3.7, and C6-1.5; (C) percent lesion length (%) for C1- 45.9, C2- 22.6, C3- 22.4, C4- 9.4, C5- 40.5, and C6- 76.7.
Patterns of root area (cm²) and disease severity (%) in four different root area classes (RACs) of ‘M.7’ rootstocks. (A) Different root area classes (RACs) observed in M.7 rootstock, and (B) Disease severity represented as percent lesion length (%) for four M.7 RACs at 8 dai.
Figure 4

A schematic representation of differential gene expression and pathway analysis.
for root-dependent fire blight infection in apple. (A) Numbers of differentially expressed genes (DEGs) obtained from analysis of control samples between RAC-1 and RAC-4, and between control and fire blight samples within RAC-1 and RAC-4. (B) Venn diagram with numbers of unique and shared DEGs from two different expression analyses. (C) Pathways showing overrepresentation from common DEGs that most likely correspond to effects of low root area and fire blight infection.

**Figure 5**

Hub genes identified from weighted gene co-expression network analysis of differentially expressed genes (DEGs). (A) Detected hub genes and their corresponding network connectivity scores as measured by degree, EPC, MCC, and MNC algorithms, Chin et al. (2014). (B) Interconnected sub-module of these hub genes, wherein different colors represent connectivities from highest (red) to lowest (yellow).

**Supplementary Files**

This is a list of supplementary files associated with the primary manuscript. Click to download.
Supporting Tables.docx
Supporting File S2.xlsx
Figure S5. Disease Progression.png
Supporting File S1.xlsx
Supporting File S3.xlsx
Supporting File S4.xlsx
Figure S3. HierarchicalClusteringb.png
Figure S6. DEGs.png
Supporting File S5.xlsx
Supporting File S6.xlsx
Figure S8. Co-Expression clusters.png
Figure S7. Pathway Genes.png
Figure S4. PCA Rotations.tif
Figure S1. ShootVariation.tif
Figure S2. Correlations_Root-Disease-RE.tif