Gene expression analysis in apple roots on soils with replant disease

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Abstract

Apple replant disease (ARD) is a serious economic risk for tree nurseries and fruit growers worldwide. ARD causes root damage and stunting in young apple trees if replanted on a spot previously occupied by the same or closely-related species, which negatively impacts yield and quality of trees and fruits. No feasible and sustainable counteraction is available up to date but understanding the apple plant's role in this complex phenomenon can help in the development of novel remedies and the early diagnosis to aid risk assessment. In this thesis, the reaction of apple rootstock plants to ARD was investigated in terms of ARD indicator gene expression in root and leaf material to identify an early and universal transcriptional marker for ARD.

Firstly, the expression of a set of previously identified candidate genes was investigated in two growth-based biotests with untreated and disinfected ARD soil. The first test included the apple genotypes M26, M9, B63 and *Malus* × *robusta* MAL0595 with differing ARD sensitivity, two ARD soils of different origin and a set of 108 candidate genes. The second test included M26, three ARD soils and seven candidate genes. These experiments revealed an early prominent induction of phytoalexin biosynthesis under ARD conditions. Two of the involved genes, *biphenyl synthase 3* (*BIS3*) and *biphenyl 4-hydroxylase b* (*B4Hb*), as well as the *ethylene-responsive transcription factor 1B-like* (*ERF1B*) were chosen for further characterization by applying a set of abiotic stressors, which revealed a response of *ERF1B* to heat stress, while *BIS3* and *B4Hb* were not affected. All three genes were induced by grass soil but to a significantly lower degree compared to ARD soil.

In the second step, we analyzed *BIS3*, *B4Hb* and *ERF1B* expression in a split-root system to simulate the plant growing in ARD and non-ARD soil at the same time. Induction of gene expression and increase of specific phenolic compounds was present in roots growing in ARD soil, accompanied by the localized ARD symptoms of root discoloration and damage. Plants partly compensated shoot growth reduction if half of the root system was growing in healthy soil. The localized response was further confirmed in a split-column system without physical barriers between ARD and healthy soil, which indicated that ARD root symptoms, gene expression and phytoalexin content depended on a direct or very close contact of ARD soil and the roots.

The defense response of increased biphenyl, dibenzofuran, phloridzin and other aromatic compound contents was observed at significantly lower but detectable levels already in healthy soil. This may play a role in the shifts observed in rhizosphere and bulk soil microbial communities leading to ARD by a gradual enrichment in specialized detrimental communities. The immobile nature of ARD enables old trees to cope with this situation by growing into soil regions not yet affected by ARD. Young trees placed in this situation, however, suffer from severe growth depression known as ARD.

Zusammenfassung

Die Apfelnachbaukrankheit (ARD) stellt ein weltweites, ernstzunehmendes ökonomisches Risiko für Baumschulen und Apfelproduzenten dar. ARD führt zu Wurzelschäden und vermindertem Sprosswachstum bei jungen Apfelbäumen, wenn sie an derselben Stelle wie dieselbe oder eine nahe verwandte Art nachgepflanzt werden. Dies führt zu Einbußen bei der Qualität der Bäume und Früchte sowie beim Ertrag. Bis jetzt gibt es keine praktikable und nachhaltige Gegenmaßnahme, doch ein besseres Verständnis der Rolle der Apfelpflanze kann zur Erarbeitung neuer Gegenmaßnahmen und Früherkennungsmethoden beitragen. In dieser Arbeit wurde die Reaktion von Apfelunterlagen auf ARD in Hinblick auf Expression von ARD-Indikatorgenen in Blatt- und Wurzelmaterial untersucht, um einen frühen und ARD-spezifischen transkriptionellen Marker für ARD zu identifizieren.

Als erstes wurde die Expression eines Sets zuvor identifizierter Kandidatengene in zwei wachstumsbasierten Biotests untersucht. Der erste Test umfasste die Apfelgenotypen M26, M9, B63 und *Malus* × *robusta* MAL0595 mit unterschiedlicher ARD-Anfälligkeit, zwei ARD-Böden und ein Set aus 108 Kandidatengenen. Der zweite Test beinhaltete M26, drei ARD-Böden und sieben Kandidatengene. In diesen Experimenten wurde eine frühe Induktion der Phytoalexinbiosynthese unter ARD-Bedingungen gefunden. Zwei der involvierten Gene, *biphenyl synthase 3* (*BIS3*) und *biphenyl 4-hydroxylase b* (*B4Hb*), sowie *ethylene-responsive transcription factor 1B-like* (*ERF1B*) wurden für die weitere Charakterisierung mittels verschiedener abiotischer Stressoren ausgewählt, bei der eine Antwort von *ERF1B* auf Hitzestress beobachtet wurde. *BIS3* und *B4Hb* waren davon nicht betroffen. Alle drei Gene waren in signifikant geringerem Maße auch in gesundem Boden im Vergleich zu ARD exprimiert.

Im zweiten Schritt wurde die Expression von *BIS3*, *B4Hb* und *ERF1B* in einem Split-Root-System untersucht, um das Wachstum der Pflanze gleichzeitig in gesundem und ARD-Boden nachzustellen. Die erhöhte Genexpression und Anreicherung spezifischer Phenole war auf Wurzeln in Kontakt mit ARD-Boden beschränkt, welche auch Verbräunungen und Wurzelschäden aufwiesen. Die Pflanzen glichen vermindertes Sprosswachstum teilweise aus, wenn eine Hälfte des Wurzelsystems in gesundem Boden wuchs. Diese lokale Antwort wurde in einem „Split-Column-System“ ohne physikalische Barrieren zwischen ARD- und Kontrollboden bestätigt. Wurzelsymptome, Genexpression und Phytoalexingehalte waren verknüpft mit einem engen Kontakt zwischen Wurzel und ARD-Boden.

Die Abwehrreaktion aus erhöhten Biphenyl-, Dibenzofuran- und Phloridzingehalten sowie Gehalten weiterer aromatischer Verbindungen fand in weit geringerem Maße aber auch in gesundem Boden statt. Dies kann eine Rolle bei den Veränderungen des Bodenmikrobioms spielen, welche durch eine schrittweise Anreicherung spezialisierter Schadorganismen zu ARD führen. Die Immobilität von ARD ermöglicht es alten Bäumen, in noch nicht betroffene Bodenbereiche vorzudringen. Werden hingegen junge Bäume in diesen Boden gepflanzt, zeigt sich die ARD-typische drastische Wachstumsverminderung.

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Supplementary information

The following information is contained as supplementary material:

1. Identical version of the doctoral thesis in electronic form (pdf)

2. File of the abstract in English and German

3. Supplementary Figures and Tables for the manuscripts:

   - **Chapter 2: Reim et al., 2020.** Genes involved in stress response and especially in phytoalexin biosynthesis are upregulated in four Malus genotypes in response to apple replant disease
   - **Chapter 3: Rohr et al., 2020.** Identification and validation of early genetic biomarkers for apple replant disease
   - **Chapter 4: Rohr et al., under review.** Split-root approach reveals localized root responses towards apple replant disease (ARD) in terms of ARD biomarker gene expression and content of phenolic compounds
   - **Chapter 5: Balbín-Suárez et al., in press.** Root exposure to apple replant disease soil triggers local defense response and rhizoplane microbiota dysbiosis

Keywords: Apple replant disease (ARD), biomarker, BioMark HD microfluidic system, gene expression, greenhouse bio-test, high throughput quantitative PCR, *Malus domestica*, *Malus* genotypes, phytoalexins, RT-qPCR, specificity, split-root experiment, soil properties, soil sickness, systemic response

1 General introduction

1.1 Apple cultivation and breeding

Apple (*Malus domestica* Borkh.) belongs to the family Rosaceae and is the most widely grown species of the genus *Malus.* Globally, it is likely the most popular fruit tree in temperate regions and is cultivated for its delicious and nutritional fruits (Ferree and Warrington, 2003). In 2018, worldwide apple production was 86.1 million metric tons, 46 % of which was produced by China (Food and Agriculture Organization of the United Nations, 2020)

Apple varieties are commonly propagated asexually by grafting due to their high heterozygosity (Goldschmidt, 2013). Triploid varieties additionally have a reproductive barrier, since three sets of chromosomes cannot be divided evenly during meiosis, resulting in aneuploids. For grafting, the rootstock is selected to convey desirable properties to the resulting tree like improved hardiness, sturdiness, and resistance against diseases and pests. Furthermore, the use of mature scions grafted onto a respective rootstock can reduce the time from planting to fruit bearing. Dwarf rootstocks can be used to produce very small trees with less than 3 m of height at maturity, which makes handling and harvest easier (van Hooijdonk et al., 2011).
Apple trees are susceptible to a number of fungal and bacterial diseases and insect pests. Commercial orchards therefore pursue plant protection programs to ensure tree health, high fruit quality and yield. Most common problems in apple cultivation are powdery mildew, codling moths, apple maggots, and several species of aphids (Way et al., 1991; Marine, 2010). Among the most serious disease problems are apple scab *(Venturia inaequalis)*, fire blight *(Erwinia amylovora)*, crown and root rot (*Phytophthora* spp.), *Gymnosporangium* rust, and canker (*Nectria* spp.) (Way et al., 1991; Vaillancourt and Hartman, 2000; Johnson, 2000; Pereira-Lorenzo et al., 2009). Breeding for resistant varieties and rootstocks is of major interest in the development of novel apple trees.

Nowadays, the locations of many single genes responsible for main characteristics and quantitative trait loci (QTL) are known. The knowledge of gene localization led to the development of a multitude of molecular markers, which are indispensable in modern apple breeding and variety identification such as isoenzymes, restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), microsatellites, amplified fragment length polymorphisms (AFLP), sequence characterized amplified regions (SCAR) and inter simple

sequence repeats (ISSR) (Pereira-Lorenzo et al., 2009 and references therein). In 2010, the genome sequence of the 'Golden Delicious' variety was published (Velasco et al., 2010) and a revised version was published in 2017 (Daccord et al., 2017).

One major obstacle remaining in apple breeding is the long-lasting juvenile stage, which lasts for 5 – 10 and even up to 12 years (Visser, 1964; Fischer, 1994). During this time, flowering and fruit production do not take place making backcrosses, inbreeding or production of new hybrids impossible (Flachowsky et al., 2009). Therefore, the production of a new apple cultivar usually takes 15 – 20 years at minimum and is very costly. A promising solution to shorten breeding cycles in apple are early flowering transgenic apple lines created by the introduction of the *BpMADS4* gene from silver birch by Flachowsky et al. (2011). Although genome engineering techniques have the potential to create minor genetic changes compared to breeding and have the potential to maintain cultivar identity, they are up to now rarely employed in apple breeding, because most quality traits are too complex to be introduced in this way.

More than 7,500 cultivars of apple are known today with a wide range of characteristics (Elzebroek and Wind, 2008). One of the most important European *Malus* germplasm resources is the gene bank located in Dresden-Pillnitz (Germany) holding more than 300 accessions of *Malus* species and hybrids, and nearly 1,000 apple cultivars from around the world (Fischer and Fischer, 1999). Different cultivars are bred for various tastes and uses, including eating raw, cooking, and juice production. Commercially popular apple cultivars are soft but crisp. Other desired qualities in modern commercial apple breeding are a colorful skin without russeting, ease of shipping and storage, high yields, and disease resistance (Sedov and Serova, 2013; Sedov et al., 2016). In apple rootstocks, breeding is mainly focused on size control (dwarfing), tolerance to low temperatures (hardiness), pest and pathogen resistance and tolerance, and adaptability to different soil conditions (Pereira-Lorenzo et al., 2009).

1.2 The worldwide impact of apple replant disease

Repeated replanting of apple and closely related rosaceous species at the same site leads to a decline in performance of the newly planted trees, which is called apple replant disease (ARD). Upon replanting, young apple trees, usually rootstocks, show a poor vegetative development, stunted shoot growth with shortened internodes and rosetted leaves, which cause a reduction in both fruit and tree quality as well as fruit yield (Caruso et al., 1989; Mazzola, 1998; Mazzola

and Manici, 2012). Belowground, ARD affected trees show root browning, discoloration and root tip necrosis, which on the microscopic level is characterized by necrotic lesions in the cortex and epidermis tissue and a reduction in functional root hairs (Hoestra, 1968; Yim et al., 2013; Yim et al., 2015; Weiß et al., 2017a; Grunewaldt-Stöcker et al., 2019).

ARD causes delays in yields by two to three years, making it an overall economic risk for fruit producers (Willett et al., 1994; Mazzola, 1998; Mazzola and Manici, 2012). This general reduced yield paired with decreased quality of the fruits causes profitability losses of 50 % estimated over the orchard's commercial life (Mazzola, 1998; van Schoor et al., 2009), which at worst can render a site unprofitable for apple cultivation (Geldart, 1994; Peterson and Hinman, 1994; Utkhede and Smith, 1994; Isutsa and Merwin, 2000). Tree nurseries are similarly or even more affected by ARD since the undesirable stunted habitus caused by it greatly reduces tree quality.

Research on symptoms, effects and causes of ARD dates back to the 1930s (e.g. Klaus, 1939; Hoestra, 1968). Hoestra (1994) characterized ARD by four main features, specificity, persistence, immobility and reversibility, which have to be taken into account when considering ARD causes and countermeasures (chapter 1.3). Specificity describes the narrow spectrum of plants affected, namely apple. Persistence and immobility mean that ARD stays in the soil over long periods of time and does not noticeably spread. Lastly, reversibility refers to the fact that an affected plant can be transferred from ARD soil to healthy soil to restore its growth. In recent years, interest in ARD research gained significance worldwide due to the overall concentration of apple cultivation to certain areas and through the use of dwarf rootstocks (Winkelmann et al., 2019). The use of these rootstocks leads to a higher planting density but also to a decrease in orchard lifetime, which in return causes a higher demand for replanting.

1.3 Causes and countermeasures of ARD

Soil disinfection experiments demonstrate the biotic nature of ARD (Yim et al., 2013; Spath et al., 2015; Weiß et al., 2017a; Weiß et al., 2017b; Reim et al., 2020). Taking studies from all over the world into consideration, ARD is caused by a complex of different pathogens. Key players of this ARD complex are parasitic fungi and oomycetes of the genera *Cylindrocarpon* (Tewoldemedhin et al., 2011a; Tewoldemedhin et al., 2011b; Franke-Whittle et al., 2015; Manici et al., 2015), *Phytophthora* (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012),

Pythium (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012; Manici et al., 2013), *Nectriaceae* (Manici et al., 2018; Popp et al., 2020) and *Rhizoctonia* (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012; Manici et al., 2013), which are enriched in ARD soil compared to healthy or disinfected soil with regional differences on a global scale. Furthermore, a decrease in beneficial organisms like fluorescent *Pseudomonas* and members of the general *Bacillus* and *Rhizophagus* has been described (Mazzola, 1999; Gu and Mazzola, 2003). Shifts in nematode communities like *Pratylenchus* have been reported. Their presence possibly enhances ARD symptoms by facilitating entryways into the root tissues for pathogens (Tewoldemedhin et al., 2011b; Galkovskyi et al., 2012; Kanfra et al., 2018). Abiotic factors such as soil texture, organic matter and pH can furthermore influence the abundance and activity of these biotic factors (Jonkers et al., 1980; Utkhede, 2006), which makes ARD an overall very complex phenomenon. There is considerable evidence that the shifts in the soil microbiome are caused by root deposits released into the soil actively by the plant via exudation or passively by decomposition of dead root material (Börner, 1959; Wittenmayer and Szabó, 2000; Hofmann et al., 2009; Winkelmann et al., 2019). The biotic nature and complexity are reflected in the most recent definition of ARD by Winkelmann et al. (2019) stating that "ARD describes a harmfully disturbed physiological and morphological reaction of apple plants to soils that faced alterations in their (micro-) biome due to previous apple cultures".

The limited mobility of ARD in the soil makes inter-row planting an option for mitigation, although this option is generally only available once. Parcel exchange or a crop rotation, which are common measures in agriculture to reduce pathogen viability naturally by the absence of a susceptible host, are not feasible in apple cultivation due to the perennial nature of the trees and the high technical investment in specialized equipment such as frost protection and irrigation systems (Winkelmann et al., 2019). Pre-planting fumigation of the soil is the most employed countermeasure against ARD (Winkelmann et al., 2019). One drawback of physical disinfection with steam however is the high energy input necessary. The alternative, chemical soil disinfection, has been carried out widely but environmental and health concerns led to a discontinuation of their registration in several European countries (Yim et al., 2013). With a lack of practicable alternatives, their use was permitted for a short period of time following strict regulations but practicable alternatives are dearly needed.

A promising integrated alternative is biofumigation, which uses the incorporation of *Brassica* plant material into the soil to achieve a similar disinfecting effect via the formation of isothiocyanates and other toxic degradation products upon decomposition (Mazzola, 1999; Yim et al.,

2013; Mazzola and Strauss, 2014; Hewavitharana et al., 2014; Yim et al., 2016; Hewavitharana and Mazzola, 2016; Yim et al., 2017; Hanschen and Winkelmann, 2020). Apple genotypes with a reduced sensitivity towards ARD have been described (Isutsa and Merwin, 2000; Reim et al., 2019) but a long-term goal is the breeding of rootstocks tolerant to ARD. To assist the development of novel strategies against ARD and breeding of less susceptible apple rootstocks, further insights into the disease etiology and the apple plant's reaction are needed (Weiß et al., 2017a; Winkelmann et al., 2019; Reim et al., 2020).

1.4 Biotests to investigate the plant's molecular reaction towards ARD

To study ARD under controlled conditions, Yim et al. (2013) developed a greenhouse biotest, which uses ARD affected soil from the field and clonally propagated plant material. The Malling rootstock M26 was selected for these tests, since it is both sensitive towards ARD and can be easily propagated in vitro (Yim et al., 2013). Shoot length and biomass reduction is used to quantify ARD severity after two to eight weeks. This short timespan allows the analysis of early reactions in juvenile trees under controlled conditions. On the other hand, quality parameters of trees and fruits relevant in assessing economic impact can only be estimated in fruit-bearing trees, which would require significantly more time.

Part of the soils used in the biotests were taken from reference sites established within the joint project BonaRes ORDIAmur (Overcoming Replant Disease by an Integrated Approach, www.bonares.de/ordiamur-de) to scientifically study ARD. These sites are situated in Heidgraben, Ellerhoop and Ruthe, cover different soil textures, climates and soil microbiome communities, but are replanted with the same known cropping history of apple since 2009 to induce ARD under controlled conditions. Plots from the same respective sites with grass cover serve as source of non-ARD soil, referred to as grass soil (Mahnkopp et al., 2018).

The sampling of comparably low soil volumes to represent a larger area relies on the assumption that soil parameters and microbial community structure are distributed homogenously across the site. Even though soil microbial community structure differs mostly on a larger scale due to soil management practices and climate (Deakin et al., 2018), recent studies uncovered considerable differences in soil microbial community structure on a small scale as well, i.e. between tree positions and the grass covered inter-row space (St. Laurent et al., 2008; Qin et al., 2016; Deakin et al., 2018). Therefore, careful sampling of the soil is vital to the validity of the biotest.

In the biotest, ARD symptoms are compared to control soil, which is either heat-disinfected (Yim et al., 2015) or gamma-irradiated ARD soil (e.g. Yim et al., 2015; Weiß et al., 2017a).

Gamma-irradiation has the advantage that it eliminates the large majority of vertebrates and invertebrates, bacteria and fungi (McNamara et al., 2003; Weiß et al., 2017a) while having a minimal effect on the soil structure compared to a heat treatment (Weiß et al., 2017a). On the downside, disinfected soil is a highly artificial system quickly recolonized by new microbiome communities, which are not native to the soil. Alternatively, grass soil of the same origin can be employed as a control (e.g. Mahnkopp et al., 2018; Lucas et al., 2018). However, studies showed that the cover crop also significantly influences soil microbial and mesofauna communities (Koehler and Born, 1989; St. Laurent et al., 2008; Deakin et al., 2018), which is why the discussion of a proper control for ARD research is ongoing (Mahnkopp et al., 2018).

Using a biotest with gamma-irradiated soils as a control, the early transcriptomic reactions of M26 plants towards ARD were studied (Weiß et al., 2017a; Weiß and Winkelmann, 2017; Weiß et al., 2017b). In these studies, first ARD symptoms of root browning were observed as early as seven days, which was the earliest time point roots and leaves for Massive Analysis of cDNA Ends (MACE) were sampled (Weiß et al., 2017a; Weiß and Winkelmann, 2017; Weiß et al., 2017b). In the roots growing in ARD soil, the analyses revealed a downregulation of primary metabolism genes and an induction of genes involved in plant defense and biotic stress response as well as regulatory and signaling genes (Weiß et al., 2017a; Weiß et al., 2017b). These studies led to the identification of a series of candidate genes with a prominent regulation in response to ARD.

Among the genes induced as part of the biotic defense, particularly genes involved in phytoalexin biosynthesis were rapidly induced in roots grown in ARD soil, which indicated a pronounced role of these components in ARD (Zhu et al., 2014; Zhu et al., 2016; Weiß et al., 2017a; Weiß et al., 2017b). Biphenyl and dibenzofuran phytoalexins are defensive compounds of the Malinae, which are known to act in the induced defense response against biotic stresses, especially pathogenic bacteria and fungi (Ahuja et al., 2012; Chizzali and Beerhues, 2012; Chizzali et al., 2012b; Chizzali et al., 2013). Induction of phytoalexin biosynthesis genes has also been observed in inoculation studies with *Pythium ultimum*, one prominent causal agent of ARD (Shin et al., 2016; Zhu et al., 2016).
Along with the induced gene expression, Weiß et al. (2017b) found an increase in phytoalexin compound content, namely 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzufuran, 2'-hydroxyaucuparin and noreriobofuran, indicating the successful translation of the induced genes and enzymatic activity of the resulting proteins. This

reaction was focused in root material, while no clear ARD specific response was found in leaf material (Weiß and Winkelmann, 2017).
Additional phenolic compounds were found to be accumulated in response to ARD soil, which may indicate increased levels of oxidative stress (Henfrey et al., 2015). The dihydrochalcones phloridzin and phloretin were found in especially high concentrations in apple root exudates and root debris, which act as reactive oxygen species scavengers and against pathogens (Hofmann et al., 2009; Emmett et al., 2014; Nicola et al., 2017; Leisso et al., 2017). Flavonol metabolism genes were also found upregulated in apple roots facing ARD soil in comparison to disinfected soil (Weiß et al., 2017a; Weiß et al., 2017b) and upon infection with *P. ultimum* (Shin et al., 2014; Zhu et al., 2014; Shin et al., 2016; Zhu et al., 2019). Furthermore, genes involved in auxin, cytokinin, ethylene and jasmonate biosynthesis and signaling were induced in roots facing ARD pathogens (Shin et al., 2014; Zhu et al., 2016; Weiß et al., 2017a; Zhu et al., 2019).

Interestingly, the plants showed severe ARD symptoms of shoot stunting and root browning when growing in the untreated ARD soil despite the high concentration of defensive compounds. Weiß et al. (2017b) hypothesized that this is an indication for a dysfunctional defense response. They theorized that the measured high concentrations of phytoalexins found in the roots might have an autotoxic effect, since high concentrations of the phytoalexin phaseolin led to cell death in *Phaseolus* and *Beta* (Glazener, 1978; Hargreaves, 1980). On the other hand, the consequences of an absence of the phytoalexin response are unknown. This could be explored in knock-out lines of genes coding for key enzymes of the biosynthesis such as *biphenyl synthase* (*BIS*) and *biphenyl 4-hydroxylase* (*B4H*) (Liu et al., 2010; Liu et al., 2007; Chizzali and Beerhues, 2012; Chizzali et al., 2012a; Weiß et al., 2017b).

Due to the immobility of ARD in the soil, in the field apple plants are faced with ARD and healthy soil simultaneously. To imitate this situation, Lucas et al. (2018) used a split-root approach where M26 plants were introduced to untreated and disinfected ARD soil or untreated ARD and healthy (grass) soil simultaneously. Lucas et al. (2018) found that ARD root symptoms of browning and biomass reduction were localized to the ARD compartments but aboveground, plants with part of the root system growing in healthy or disinfected soil were able to compensate shoot growth partially. This confirms that ARD is affecting the plant locally. Little is however known about the transcriptional reactions inside the plant in a split-root system.

1.5 Thesis objectives

This thesis follows the findings of Weiß et al. (2017a; 2017b) in investigating the early expression of selected ARD responsive genes in apple. We aimed at identifying genes that are early indicators for ARD indicated by their expression pattern. These biomarker genes would then shed more light on the ARD etiology, which in return can help in an early ARD diagnosis and breeding of ARD tolerant apple rootstocks. This endeavor was separated into two major goals: identifying marker genes as early indicators for ARD on a transcriptional level (chapters 2 and 3) and localizing the molecular response of apple within the root system using these biomarker genes in a split-root and split-column approach (chapters 3 and 4).

Firstly, we aimed at narrowing down the set of candidate genes identified by Weiß et al. (2017a; 2017b). A set 108 candidate genes derived from the studies of Weiß et al. (2017a; 2017b) and the literature was investigated in root and leaf material of the three ARD sensitive apple genotypes M26 and M9, the rootstock of unknown ARD reaction B63 and the ARD tolerant accession *Malus* × *robusta* MAL0595 (Reim et al., 2019). Gene expression was analyzed after growing the plants for 7 days in gamma-irradiated or untreated ARD soil from the apple growing area of Meckenheim or the ORDIAmur reference site Heidgraben. Here, we aimed at identifying genes with a consistent response among the two ARD soils tested but with differences between the genotypes correlated to ARD tolerance. This experiment and its findings are summarized within Reim et al. (2020, chapter 2).

Then, using biotests with plants of apple rootstock M26 and three ARD soils from the reference sites Ellerhoop, Heidgraben and Ruthe (each untreated and gamma-irradiated) the expression of seven candidate genes derived from the previous studies (Weiß et al., 2017a; Weiß et al., 2017b) was analyzed in the roots after 1, 3, and 7 days. Thereby it was intended to achieve an even earlier resolution in gene expression than investigated by Weiß et al. (2017b). To yield a final selection on ARD biomarker indicator genes, the resulting promising candidate genes showing an early and consistent regulation in all three ARD soils were then further characterized for ARD specificity with abiotic stressors. These experiments are described in the manuscript entitled “Identification and validation of early genetic biomarkers for apple replant disease” (Rohr et al., 2020, chapter 3).

In the second step, we aimed to employ the ARD biomarker genes identified in the first step to further investigate the localized molecular plant response towards ARD. These experiments

followed the experiential setup of Lucas et al. (2018), who found root growth locally diminished upon exposure to ARD soil. In two experiments, we analyzed the expression of the three most promising candidate genes from the preceding experiments, *biphenyl synthase 3*, *biphenyl 4-hydroxylase b* and *ethylene-responsive transcription factor 1B-like*, and connected their expression to plant growth above and belowground. Using soil from the two sites Heidgraben and Ellerhoop a further objective was to analyze the contents of selected phenolics next to gene expression. The results are presented in the manuscript "Split-root approach reveals localized root responses towards apple replant disease (ARD) in terms of ARD biomarker gene expression and content of phenolic compounds" (Rohr et al., under review, chapter 4).

In the split-root system, we investigated biomarker gene expression and content of phenolic compounds in response to the ARD affected roots, which we aimed to further narrow down in a split-column system without physical barriers. Since the plants were allowed to grow freely in this system, the expression of our ARD biomarker genes could be observed in the same roots growing from control soil into ARD soil, which allowed for an in depth localization of the gene expression response. The experiment is summarized in "Root exposure to apple replant disease soil triggers local defense response and rhizoplane microbiota dysbiosis" (Balbín-Suárez et al., in press, chapter 5).

2 Genes involved in stress response and especially in phytoalexin biosynthesis are up-regulated in four *Malus* genotypes in response to apple replant disease

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Genes Involved in Stress Response and Especially in Phytoalexin Biosynthesis Are Upregulated in Four *Malus* Genotypes in Response to Apple Replant Disease

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Apple replant disease (ARD) is a soil-borne disease, which is of particular importance for fruit tree nurseries and fruit growers. The disease manifests by a poor vegetative development, stunted growth, and reduced yield in terms of quantity and quality, if apple plants (usually rootstocks) are replanted several times at the same site. Genotype-specific differences in the reaction of apple plants to ARD are documented, but less is known about the genetic mechanisms behind this symptomatology. Recent transcriptome analyses resulted in a number of candidate genes possibly involved in the plant response. In the present study, the expression of 108 selected candidate genes was investigated in root and leaf tissue of four different apple genotypes grown in untreated ARD soil and ARD soil disinfected by γ-irradiation originating from two different sites in Germany. Thirty-nine out of the 108 candidate genes were differentially expressed in roots by taking a p-value of < 0.05 and a fold change of > 1.5 as cutoff. Sixteen genes were more than 4.5-fold upregulated in roots of plants grown in ARD soil. The four genes *MNL2* (putative mannosidase); *ALF5* (multi antimicrobial extrusion protein); *UGT73B4* (uridine diphosphate (UDP)-glycosyltransferase 73B4), and *ECHI* (chitin-binding) were significantly upregulated in roots. These genes seem to be related to the host plant response to ARD, although they have never been described in this context before. Six of the highly upregulated genes belong to the phytoalexin biosynthesis pathway. Their genotype-specific gene expression pattern was consistent with the phytoalexin content measured in roots. The biphenyl synthase (*BIS*) genes were found to be useful as early biomarkers for ARD, because their expression pattern correlated well with the phenotypic reaction of the *Malus* genotypes investigated.

Keywords: apple replant disease (ARD), gene expression, BioMark HD microfluidic system, high-throughput qRT-PCR, phytoalexins, greenhouse bio-test, soil properties, *Malus* genotypes

INTRODUCTION

Replanting apple trees at a site previously occupied by an apple plant leads to stunted shoot growth with shortened internodes, root damage, root tip necrosis, and reduction of functional root hairs (Caruso et al., 1989; Mazzola, 1998; Mazzola and Manici, 2012; Yim et al., 2013; Grunewaldt-Stöcker et al., 2019). These symptoms are referred to as apple replant disease (ARD). ARD represents a serious economic risk for tree nurseries and orchards as it leads to decreased and delayed fruit yields and reduced fruit and tree quality (Mazzola, 1998; Mazzola and Manici, 2012). At worst, a site strongly affected by ARD may become unprofitable for further apple cultivation (Geldart, 1994; Peterson and Hinman, 1994; Utkhede and Smith, 1994; Isutsa and Merwin, 2000).

Biotic agents represent the main causes of ARD as demonstrated by various disinfection experiments (Mazzola, 1998; Yim et al., 2013; Spath et al., 2015). Winkelmann et al. (2019) defined ARD as "a harmfully disturbed physiological and morphological reaction of apple plants to soils that faced alterations in their (micro)biome due to previous apple cultures." There is substantial evidence that the changes in the soil biota trace back to root exudates and material from decomposing apple roots (Börner, 1959; Wittenmayer and Szabó, 2000; Hofmann et al., 2009; Winkelmann et al., 2019). Up to now, no practicable counteraction against ARD is available. The most employed countermeasures, crop rotation and soil disinfection, are unfeasible due to either environmental hazards or high costs (Winkelmann et al., 2019). In order to develop novel strategies against ARD, both the reactions of the apple plant and the etiology of the disease should be understood in more detail (Weiß et al., 2017b; Winkelmann et al., 2019).

Recent transcriptomic analyses revealed the induction of genes associated with biotic stress response in roots of apple plants grown in ARD soil (Weiß et al., 2017a; Weiß et al., 2017b). This corresponds well with the findings, that parasitic fungi and oomycetes of the genera *Cylindrocarpon* (Tewoldemedhin et al., 2011b; Mazzola and Manici, 2012; Franke-Whittle et al., 2015; Manici et al., 2015), *Phytophthora* (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012), *Pythium* (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012; Manici et al., 2013), and *Rhizoctonia* (Tewoldemedhin et al., 2011a; Mazzola and Manici, 2012; Manici et al., 2013) are enriched in ARD soil in comparison to healthy or disinfected soil. In particular, genes of the biphenyl biosynthetic pathway were rapidly activated in the roots of apple plants grown in ARD soil (Zhu et al., 2014; Zhu et al., 2016; Weiß et al., 2017a; Weiß et al., 2017b). Phytoalexins like biphenyls and dibenzofurans are known to act in an induced defense mechanism against biotic stressors, such as fungi and bacteria (Ahuja et al., 2012; Chizzali et al., 2012b; Chizzali and Beerhues, 2012; Chizzali et al., 2013). Interestingly, the activation of phytoalexin biosynthesis genes was also found when the plants were inoculated with *Pythium ultimum*, one component of the ARD complex (Shin et al., 2016b; Zhu et al., 2016). Along with the elevated gene expression, individual phytoalexin compounds were found in higher abundances in roots growing in ARD soils (Weiß et al., 2017b).

Additional phenolic compounds play a role in the ARD response, which were shown to accumulate in response to infected soil and may indicate the occurrence of oxidative stress (Henfrey et al., 2015). Especially the dihydrochalcones phloridzin and phloretin have been found highly abundant in apple root exudates and root debris (Hofmann et al., 2009; Emmett et al., 2014; Nicola et al., 2016; Leisso et al., 2018). They act against pathogens and as scavengers of reactive oxygen species (Börner, 1959; Emmett et al., 2014; Henfrey et al., 2015). An upregulation of flavonol metabolism genes was also found in apple roots under replant conditions (Weiß et al., 2017a; Weiß et al., 2017b) and upon infection with *P. ultimum* (Shin et al., 2014; Zhu et al., 2014; Shin et al., 2016b; Zhu et al., 2019).

Further genes upregulated under ARD conditions are involved in auxin, ethylene, jasmonate, and cytokinin biosyntheses and signaling (Shin et al., 2014; Shin et al., 2016b; Weiß et al., 2017a; Zhu et al., 2019). Salicylic acid, ethylene, and jasmonic acid are important signaling compounds in the biotic stress defense response (Glazebrook, 2005; Broekaert et al., 2006). Moreover, ethylene can induce the biosynthesis of phytoalexins derived from the phenylpropanoid pathway (Kamo et al., 2000; Chung et al., 2001; Ishigaki et al., 2004). Biotic stress signaling involves the activation of signal transduction pathways and the activation of a number of transcription factors. As plant shoot and root growth are strongly altered by ARD, changes in auxin, cytokinin, abscisic acid, and gibberellin homeostasis and signaling are expected to occur.

In this study, we compared the expression of 108 candidate genes (CGs) that were supposed to be involved in the reaction of apple to ARD soil. The majority of these CGs were selected from the transcriptomic data available from Weiß et al. (2017a 2017b)) and Weiß and Winkelmann (2017). These CGs were shown to be differentially expressed in roots and leaves of the ARD-sensitive apple rootstock M26 grown in untreated ARD soil and disinfected ARD soil. Further CGs were chosen based on the literature with a focus on the following functional categories: flavonoid biosynthesis, oxidation–reduction processes, jasmonic acid–mediated signaling and responses to wounding, defense, and auxin metabolism (Dal Cin et al., 2009; Milcevicova et al., 2010; Devoghalaere et al., 2012; Dugé De Bernonville et al., 2012; Shin et al., 2014; Qian et al., 2016; Shin et al., 2016a). CG expression was compared between four apple genotypes with different genetic background and different susceptibility/tolerance towards ARD. The apple genotypes were grown in a bio-test using ARD soil from two different ARD sites.

The objectives of the present study were: (I) to evaluate the expression of 108 CGs in response to ARD in roots and leaves of plants tested in a greenhouse bio-test using a high-throughput microfluidic approach, (II) to determine the influence of the *Malus* genotype on the quantitative expression of the CGs, and (III) to correlate the gene expression data to both the ARD severity measured in the bio-test employing two different ARD soils and the phytoalexin contents detected in roots. The results provide new insights into genotypic differences in the complex reaction to ARD and give new hints to mechanisms contributing to ARD sensitivity or tolerance.

MATERIAL AND METHODS

Soil Origin and γ-Irradiation

ARD soil from the two sites Heidgraben (53°41'57.5"N, 9° 40'59.6"E) and Meckenheim (50°37'8.5"N, 6°59'25.4"E) was sampled at a depth of 0–20 cm. The soil from Heidgraben is an entic podzol, and that from Meckenheim was classified as a haplic luvisol developed from loess (Mahnkopp et al., 2018). The detailed soil properties are described in **Table 1**. On the sampled Heidgraben plots, ARD had been induced by four times replanting of *Malus domestica* Borkh. cv. 'Bittenfelder' as described in detail by Mahnkopp et al. (2018). The Meckenheim site has been in use for apple variety tests grafted on the rootstock M9 since 2006. Replanting took place in the years 2010 and 2017 (G. Baab and L. von Schoenebeck, personal communication).

Both soils were sieved through an 8 mm mesh. Half of each soil volume was filled into autoclavable bags in portions of 10–15 L. The soil was γ-irradiated with a minimum dose of 10 kGy (recorded dosages: minimum 10.87 kGy, maximum 31.96 kGy, BGS Beta-Gamma-Service, Wiehl, Germany) by which most fungi, bacteria, and invertebrates are killed (McNamara et al., 2003). Hereafter, the untreated ARD soil will be denoted as ARD soil and the ARD soil disinfected by γ-irradiation as γARD soil.

The effect of the γ-irradiation was confirmed by plating diluted soil solutions on growth media selective for bacterial or fungal growth (Balbín-Suárez et al., personal communication). Bacterial colony-forming units (CFUs) were counted after 2 days and fungal CFUs after 7 days. Briefly, 9 mL of 0.85% NaCl solution (saline) were added to 1 g of soil under sterile conditions and vortexed for 2 min. After settling of the soil particles, serial dilutions (factor 10) of the supernatant were made by mixing 100 µL soil solution with 900 µL saline. For each of the four soil variants, two samples were taken for plating. For the γ-irradiated soil samples, 100 µL of the 1:10 and 1:100 dilution were plated; for the untreated soil variants, dilutions 1:100, 1:1,000, and 1:10,000 were plated. Each plating was carried out in triplicates. The culture media used were Reasoner's 2A agar (R2A agar, Carl Roth, Karlsruhe, Germany) supplemented with 100 mg L^{-1} cycloheximide for bacteria and Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) supplemented with 100 mg L^{-1} penicillin, 10 mg L^{-1} tetracycline, and 50 mg L^{-1} streptomycin for fungi. The plating was carried out twice, before and after storage of the γ-irradiated and untreated soils, to evaluate an effect of the storage (**Table S3**).

TABLE 1 | Major properties of soils from the two apple replant disease (ARD) sites Heidgraben and Meckenheim at 0–20 cm depth.

Site	Particle size distribution			SOC [g kg^{-1}]	N_{total} [g kg^{-1}]	pH ($CaCl_2$)	$CaCO_3$ [g kg^{-1}]
	Sand [%]	Silt [%]	Clay [%]				
Heidgraben	92.9	2.8	3.1	25.4	1.54	5.3	<0.1
Meckenheim	6.9	72.0	21.1	12.3	1.5	6.7	<0.1

Particle size distribution, total carbon and nitrogen (N_{total}) are displayed. Soil organic carbon (SOC) represents total carbon due to the absence of carbonate ($CaCO_3$).

Plant Material and Experimental Setup

Plants of the apple genotypes M26, M9, B63, and the *Malus* × *robusta* accession MAL0595 were used. B63 is an offspring of the cross (*M. purpurea* 'Eleyi' × *M. sieboldii*) × M9 and was derived from a breeding program for resistance to apple proliferation disease (W. Jarausch, personal communication). The accession MAL0595 was derived from the *Malus* gene bank collection of the Julius Kühn-Institut (Dresden-Pillnitz, Germany). All genotypes were propagated *in vitro* via axillary shoots on a modified MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.5 µM indole-3-butyric acid (IBA), and 4.4 µM 6-benzylaminopurine (BAP). For M9, 2 mL L^{-1} Plant Preservative Mixture (PPM, Plant Cell Technology, Washington DC, USA) was added to the culture medium in order to control growth of endophytic bacteria. MAL0595 subculture was carried out once with MS medium containing 3% sucrose, 0.5 µM IBA, and 4.54 µM Thidiazuron (TDZ) to increase the number of shoots obtained. All *in vitro* cultures were incubated at 24°C with a 16 h photoperiod provided by Philips MASTER TL-D 58W/865 fluorescence tubes at a PPFD (Photosynthetic Photon Flux Density) of 35–40 µmol m^{-2} s^{-1}.

In vitro rooting was induced by transferring the 5-week-old shoots to ½ MS medium supplemented with 2% sucrose and 4.92 µM IBA (Weiß et al., 2017a). The rooting percentages determined 2 weeks after transfer to rooting medium were 95.8% for M26 (n = 168), 8.9% for M9 (n = 168), 64.8% for B63 (n = 168), and 31.4% for MAL0595 (n = 242) respectively. All plants were transferred to peat substrate (Steckmedium, Klasmann-Deilmann GmbH, Geeste, Germany). For acclimatization, the shoots were cultivated under covers to ensure high humidity. During acclimatization, the plants were adapted to greenhouse conditions by gradually reducing the air humidity. After about 4 weeks, the plants were introduced to the bio-test. ARD and γARD soils from Heidgraben and Meckenheim were supplemented with 2 g L^{-1} of the slow-release fertilizer Osmocote Exact 3-4 M (16-9-12+2MgO+trace elements, https://icl-sf.com) and filled into fifteen 0.4 L pots for gene expression samples and ten 1 L pots per soil variant and genotype for growth parameters. The M9 rootstock was tested with only 12 plants due to poor rooting and acclimatization, 6 in Heidgraben ARD soil and 6 in γARD soil in 0.4 L pots for gene expression analysis.

The greenhouse experiment took place from August 9, 2017, to September 7, 2017, at the campus Herrenhausen (Gottfried Wilhelm Leibniz University Hannover, Hanover, Germany). All 312 plants were randomly arranged and cultivated at 22.4 ± 2.8°C and a relative air humidity of 68.2 ± 8.2%. Additional light was provided whenever solar irradiation fell below 25 klx to provide 16 h of daylight. Plant protection against thrips was carried out according to horticultural practice. Shoot length was measured on a weekly basis.

After 7 days, all plants for gene expression analysis were harvested and carefully removed from the soil. Whole root systems were washed gently in tap water and dried with paper towels, and the three youngest fully developed leaves were

sampled. Root and leaf samples were transferred to 2 mL reagent tubes, immediately frozen in liquid nitrogen, and stored at −80°C until RNA isolation.

Ten plants per variant representing 10 biological replicates (except of M9) were harvested after 4 weeks for determining growth parameters. Single plants died off (resulting in only nine biological replicates) from the following variants (**Table S1**): MAL0595 in Heidgraben ARD, B63 in Meckenheim ARD, and MAL0509 in Meckenheim γARD. Plant quality was assessed visually by inspection of root color and habitus. Plant growth was determined by measuring shoot length as well as shoot and root fresh masses. Roots of four to five plants per variant were lyophilized for 3 days and used for dry mass evaluation and phytoalexin analysis after freeze-drying for 3 days.

RNA Isolation and First Strand cDNA Synthesis

From 15 plants of each of the genotypes M26, B63, and MAL0595, five pools containing 3 single plants each, i.e. five biological replicates, were established for each of the four soil variants. For M9, only 12 plants were available. These plants were grown in Heidgraben soil, six in γARD soil and six in ARD soil, respectively. For M9, two pooled samples containing three plants each were created for each of the two soil variants. Selection of the plants for each pool was carried out with regard to shoot length to achieve a similar mean shoot length among the pools.

The pooled samples were homogenized in a Mixer Mill at 27 Hz for 1 min (Mixer Mill MM400, Retsch, Haan, Germany) cooled with liquid nitrogen. Total RNA was extracted from 100 mg of frozen ground plant material with RP lysis buffer using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) according to the manufacturer's instructions. Genomic DNA was removed with DNase I (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA concentration and quality was determined spectrophotometrically (NanoDrop 2000c, Peqlab, Erlangen, Germany). The integrity was checked on a 1% agarose gel. The isolated RNA was stored at −80°C until first strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) together with oligo dT primers and 1 µg RNA as template. The resulting cDNA was diluted 10-fold in nuclease-free water and stored at −20°C until use. The success of cDNA synthesis and the exclusion of genomic DNA contaminations was verified in a standard PCR with the primer pair EF1-for/-rev (EF1-for ATTGTGGTCATTG GYCAYGT; EF1-rev CCAATCTTGTAVACATCCTG) using 1 µl of the diluted cDNA as well as 1 µl of the RNA preparation (Boudichevskaia et al. (2009). PCR products resulting from genomic DNA and cDNA differ in fragment size (905bp/ 707bp), whereas no product should be generated using RNA.

Primer Selection and RT-qPCR Validation

The CG primer set was compiled on the basis of genes differentially expressed in root and leaf material of *Malus* rootstock M26 grown in ARD soil compared to γARD soil (Weiß et al. 2017a; Weiß et al. 2017b; Weiß and Winkelmann, 2017). Additionally, known pathogen and stress-related genes focusing on plant hormone signaling of *Malus* and *Arabidopsis thaliana* described in the literature were selected (Dal Cin et al., 2009; Milcevicova et al., 2010; Devoghalaere et al., 2012; Dugé De Bernonville et al., 2012; Shin et al., 2014; Qian et al., 2016; Shin et al., 2016a; Weiß et al., 2017a; Weiß et al., 2017b). A full list of all primers is provided in **Table S2**. All primers were validated *in silico* using the software program FastPCR v6.6 (PrimerDigital Ltd, Helsinki, Finland) (Kalendar et al., 2017) by calculating theoretical PCR results using the *Malus* × *domestica*.v1.0. consensus_CDS database obtained from http://www.rosaceae.org. The program predicted possible PCR products with a length of 50–3,000 bp, with one mismatch allowed at the 3'-end.

New primers were designed using the Primer3 web tool with the following parameters: primer length 18–24 bp, amplification product 100–200 bp, T_M = 59–61°C, CG content 40–60%. The specificity of the new primers was also tested *in silico* as described. Primer sequences with proven specificity to the target gene sequence were checked for sufficient amplification efficiency with RT-qPCR. The *Elongation factor 1-α* [MDP0000304140], *Elongation factor 1β-like* [MDP0000903484], *Tubulin beta chain* [MDP00009551799], *Ubiquitin-conjugating enzyme E2 10-like* [MDP0000140755], and *Actin-7* [MDP0000774288] were used as reference genes according to Weiß et al. (2017a). Each primer combination (75 nM each primer) was analyzed with three technical replicates using the Maxima SYBR Green master mix (Thermo Fisher Scientific, Schwerte, Germany). All primers were tested at an annealing temperature of 60°C and cDNA of the apple rootstocks M9, M26, CG41 and the wild apple genotype *Malus* × *robusta* 5 (accession no. MAL0991) grown in untreated ARD soil, since no cDNA of B63 and MAL0595 was available at this time. RT-qPCR was performed on an iCycler iQ Real Time PCR Detection System (Bio-Rad) with an initial denaturation of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The PCR products were analyzed by melt-curve analysis of 55°C to 80°C with an increment of 0.5°C for 10 s each step. Data were recorded with the software package Genex (Bio-Rad, München, Germany). PCR efficiencies were calculated using the software program LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). The PCR efficiencies presented in **Table S2** are mean values of all samples per primer combination, where expected amplicons (based on melting temperature) were detectable. Primer pairs producing more than one distinct peak in the melt-curve analysis were assigned as not specific. These primers were rejected from further RT-qPCR analysis.

Expression Analysis Using Quantitative PCR

RT-qPCR was performed using the BioMark HD high-throughput system (Fluidigm, South San Francisco, California, USA) by analyzing 128 individual samples, consisting of 64 root and leaf samples respectively, with 116 primer pairs (including 5 primer pairs for reference genes) using six Dynamic Array™ integrated fluidic circuits (96.96 IFCs, Fluidigm, South San Francisco, California, USA). The sample design included five biological replicates for each genotype (B63, M26 and

MAL0595), soil treatment, and soil origin. For M9, only samples of the Heidgraben soil were analyzed with only two replicates of each soil treatment (ARD/γARD). The entire analysis included two technical repetitions for each biological replicate. Default space on these IFCs allowed the analysis of 96 samples with 96 primers in one run.

For specific target amplification, 1.25 µL cDNA was pre-amplified in a mixture with 0.5 µL of pooled primers (final concentration, 500 nM), 2.5 µL of 2× PreAmp Master Mix (Applied Biosystems, Carlsbad, CA, USA), and 0.75 µL of water. The cycling program was as follows: 95°C for 10 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min. Afterwards, the PCR reactions were purified with exonuclease (20 U μL^{-1}) and diluted 1:5 with Teknova-DNA suspension buffer (VWR, Darmstadt, Germany). The qPCR was performed in 96.96 Dynamic Array™ IFCs (Fluidigm, South San Francisco, CA, USA) following the manufacturer's instructions. Each assay inlet contained 5 µL of an assay mix consisting of 0.5 µM primer mix, 2.5 µL assay loading reagent (Fluidigm), and 2.25 µL 1× TE buffer assay reagent. The Fluidigm sample premix contained 2.25 µL of the pre-amplified sample, 2.5 µL of 2× SsoFast EvaGreen supermix with low ROX (Bio-Rad, München, Germany), and 0.25 µL of 20× Binding Dye Sample Loading Reagent (Fluidigm). The cycling program was: 1 min at 95°C, followed by 30 cycles of 96°C for 5 s and 20 s at 60°C plus melting curve analysis.

Extraction and Analysis of Phytoalexins

At the final evaluation of the experiment (4 weeks after potting), the root systems of the genotypes M26, B63 and MAL0595 were combined to obtain two pools (i.e. two biological replicates composed of roots of four to five plants) per soil variant. The roots were lyophilized for 3 days (alpha 1-2 LDplus, Christ, Osterode, Germany). The dry roots were homogenized in a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) with steel beads. Before phytoalexin extraction, 4-hydroxybiphenyl (50 µg) was added to each sample (around 100 mg DW each) as internal standard for quantification in gas chromatography–mass spectrometry (GC-MS) measurement. The samples were extracted with 1 mL methanol by shaking in a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) for 20 min. The extracts were centrifuged at room temperature at 13,000 rpm for 10 min. An aliquot of the supernatant (200 µL) was transferred to a new 1.5 mL Eppendorf tube and dried under a constant air stream. The residue was re-suspended in 200 µL ethyl acetate and centrifuged at 13,000 rpm for 10 min. The resulting clear supernatant was transferred to a GC-MS vial with a glass inlet. After removal of the ethyl acetate by air stream, 50 µL N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) was added to the inlets for derivatization at 60°C for 30 min. The samples were then measured by GC-MS, as described previously (Hüttner et al., 2010).

Data Analysis and Statistical Evaluation

A mean PCR efficiency (quality score) was calculated using the Fluidigm Real-Time PCR Analysis Software v4.3.1 (Fluidigm, South San Francisco, CA, USA). Therefore, each individual amplification curve was compared to an ideal exponential curve. The closer the amplification curve is to the ideal, the quality score approaches 1. The further the curve is from ideal, the quality score approaches 0. Only quality score values above 0.65 (an arbitrary threshold set by Fluidigm) passed the quality check. Curves that fail the quality threshold were excluded from further calculations. Considering the quality threshold and the quantification cycle (Cq), separate ΔCq values for sample and control were calculated. This was done on basis of the following formulas:

$$\text{Sample} \quad \Delta Cq = \Delta Cq \text{ Candidate gene (ARD soil)} - \Delta Cq \text{ reference gene (ARD soil)}$$

$$\text{Control} \quad \Delta Cq = \Delta Cq \text{ Candidate gene } (\gamma\text{ARD soil}) - \Delta Cq \text{ reference gene } (\gamma\text{ARD soil})$$

The reference genes were validated according to their stability using NormFinder (Andersen et al., 2004). All reference genes with stability values below 0.25 were included in the ΔCq value calculation, so that depending on the IFC, three to five reference genes were considered in the control ΔCq calculation. The ΔΔCq value was calculated by subtracting the control ΔCq value from the sample ΔCq value, which resulted in the relative gene expression (fold change, $2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001). Throughout this paper, gene expression is presented as relative expression level in ARD soil compared to the expression in γARD soil, which was set to be one.

The test for normal distribution was carried out with the Shapiro–Wilk test using SAS version 9.4 (SAS, NC, USA). The effect on gene expression of different soil treatments (ARD soil and γARD soil) was tested with the analysis of variance (ANOVA) also using SAS version 9.4. Furthermore, the effect of genotype and soil origin (type) on gene expression was tested using the ANOVA procedure MIXED in SAS version 9.4. The STRING database (Szklarczyk et al., 2017) was used to predict the interaction of the detected differentially expressed genes (DEGs).

Data on shoot length, fresh and dry masses, and phytoalexin content were evaluated using R version 3.5.1 (R Development Core Team, 2011) in R Studio version 1.1.45. The data were checked for a Gaussian distribution and log transformed, if necessary. A linear model was fitted for each parameter, and an ANOVA was calculated. Multiple comparisons of means (Tukey test) were carried out using the R package "multcomp" version 1.4-8 (Hothorn et al., 2008).

Using the software program SAS version 9.4. Pearson's correlation was analyzed between the phenotypic data (shoot length and fresh biomass) and the fold change values of selected phytoalexins as well as CGs.

RESULTS

Phenotyping of the Genotypes After 4 Weeks

Plating of the soil solution proved the success of the soil disinfection with the significant reduction in bacterial and

fungal colony-forming units (CFUs) (**Table S3**). Plant growth of the genotypes M26, B63, and MAL0595 was negatively affected by ARD. After 4 weeks of cultivation in ARD soil, shoots were smaller with lower biomasses for B63 and M26, but not for MAL0595 (**Tables 2** and **S2**). The reduction in shoot length was stronger in Meckenheim soil than in Heidgraben soil (**Table 2**).

As depicted in **Figure 1**, the roots of all three genotypes showed a darker coloration when grown in ARD soil from both sites. In addition, less fine roots were visible in the ARD variants. The rootstock M9 was not included in this final evaluation, as only a few plants were available.

Shoot and root fresh biomass of B63 and M26, were significantly reduced on ARD soil from both sites. For MAL0595, the change in fresh biomass was not significant. In Meckenheim soil, M26, and B63 showed a stronger reduction in shoot and root biomass compared to Heidgraben soil (**Table 2**). Generally, a higher shoot fresh biomass was achieved by plants grown in Meckenheim soil as seen by the control plants grown in γARD soil from this site. A similar pattern was found for the fresh root biomasses. A significant reduction was observed for M26, with a stronger effect in Meckenheim soil. MAL0595 root biomass did not differ significantly between the treatments, and in Meckenheim soil, the reduction was approximately halved compared to B63 and M26, (**Table 2**). For B63, root biomass was not significantly reduced when grown in Meckenheim ARD soil.

Although MAL0595 showed comparable reduction in shoot and root biomass with B63 and M26 (except root biomass in Meckenheim soil), this reduction was statistically not significant. One explanation for that is the high variation between individual plants from the same genotype.

Establishment of Gene-Specific Primers

Primer pairs for 122 genes (117 CGs and 5 reference genes) were tested *in silico* against the *Malus* × *domestica*.v1.0.consensus_CDS database (**Table S2**). Thirty-nine combinations showed unspecific amplification. Redesign of new primer combinations was successful for 33 out of these genes. For six genes, no gene-specific primers were found. These genes were excluded from subsequent analyses (**Table S4**). In total, 111 primer pairs (CGs only) were tested for their amplification efficiency by RT-qPCR (**Table S2**). The PCR efficiencies varied between 1.77 and 2.10 (a value of 2 is equal to an amplification efficiency of 100%). After melt-curve analysis, 108 primer combinations were confirmed as highly specific, whereas the specificity of three combinations (*IPT*, *Mal d1.06*, and *FGT*) was insufficient. For four primer combinations (*NTL9*, *PDF2.2*, *ABCB11b*, and *Bax_inh*) the melting temperature varied slightly. These ranges were detectable between individual samples of the same tissue of the same genotype. On this account, the amplicons were most likely derived from the same gene and not from different orthologous sequences.

Genes Differentially Expressed in Response to ARD

Gene expression of 108 CGs was analyzed in leaf and root tissue of B63, M26, M9, and MAL0595. For this, plant material was collected after 7 days of cultivation in four different soil variants. The relative gene expression (ARD soil vs. γARD soil) ranged from 0.5-fold to 31.9-fold (**Table S5**). Fourteen CGs were slightly downregulated in plants grown in ARD soil compared to those grown in γARD soil (10 genes in roots, 3 in leaves, and 1 in leaves and roots). Out of the 108 CGs, 42 DEGs were identified by taking a p-value of < 0.05 and a fold change of greater than 1.5 as cutoff (**Table S6**). Thirty-nine genes were differentially expressed in roots. Thirty-one of them were only differentially expressed in roots, whereas eight genes were upregulated in both tissues. The remaining four genes were differentially expressed in leaves only.

TABLE 2 | Shoot length and fresh mass of shoot and root of M26, B63, and MAL0595 4 weeks after transplanting to γARD soil and ARD soil from Heidgraben and Meckenheim.

Genotype	Shoot length	Heidgraben				Meckenheim			
		γARD soil	**ARD soil**	**% red**		**γARD soil**	**ARD soil**	**% red**	
B63	[cm]	4.1 b	2.8 a	**–32.4**	**	7.4 c	3.0 a	**–60.2**	***
M26		3.5 b	2.5 a	**–30.0**	*	5.9 c	3.6 b	**–39.8**	***
MAL0595		5.1 a	4.5 a	**–11.6**	n.s.	6.2 a	4.4 a	**–28.0**	n.s.
Genotype	Fresh biomass **shoot**	**Heidgraben**				**Meckenheim**			
		γARD soil	**ARD soil**	**% red**		**γARD soil**	**ARD soil**	**% red**	
B63	[g]	0.95 b	0.54 a	**–42.8**	**	1.40 c	0.71 ab	**–49.3**	***
M26		1.00 b	0.60 a	**–39.8**	*	1.57 c	0.81 ab	**–48.1**	***
MAL0595		0.81 ab	0.48 b	**–41.2**	n.s.	1.47 a	0.88 ab	**–39.9**	n.s.
Genotype	Fresh biomass **root**	**Heidgraben**				**Meckenheim**			
		γARD soil	**ARD soil**	**% red**		**γARD soil**	**ARD soil**	**% red**	
B63	[g]	0.42 b	0.24 a	**–41.3**	*	0.35 ab	0.21 a	**–37.8**	n.s.
M26		0.27 c	0.16 ab	**–40.2**	**	0.23 bc	0.12 a	**–48.2**	***
MAL0595		0.36 a	0.20 a	**–42.7**	n.s.	0.31 a	0.36 a	**19.5**	n.s.

Mean values with the same letter within one row did not differ significantly (Tukey test, p = 0.05, n = 9–10). Reduction (%, red) compared between γARD soil and ARD soil from one site is given in bold. Asterisks indicate a significant reduction regarding the Tukey test [p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***); n.s. = not significant].

FIGURE 1 | Apple plants of M26, B63 and MAL0595 4 weeks after planting to γ-irradiated apple replant disease (γARD) soil and ARD soil from the sites Heidgraben and Meckenheim.

Highly Regulated CGs with a Significant Fold Change > 4.5

Sixteen CGs were highly, i.e. more than 4.5-fold, upregulated in roots of plants growing in ARD soil compared to those growing in γARD soils. Fourteen of them were significantly upregulated in root tissue in all four apple genotypes (**Figure 2**). Six of them (*BIS1, BIS2, BIS3, BIS4, B4Ha,* and *B4Hb*) belong to the phytoalexin biosynthetic pathway, whereas one gene (*ERF1B*) is a transcription factor binding to a pathogenesis-related element and an additional gene belongs to the endochitinase family (*CHIB*). The six remaining genes are associated with six gene families of different biological functions (**Figure 3**). The highest upregulation of gene expression in roots grown in ARD soils was detected for the phytoalexin biosynthesis genes. The average fold changes were 31.9 for *BIS4*, 27.8 for *BIS1*, and 24.0 for *BIS2*. In contrast, *BIS3* was only 8.8 times more highly expressed in ARD soil than in γARD soil, but this gene showed the overall highest expression level (**Figure 2** and **Table S5**). The two further genes of this pathway, *B4Ha* and *B4Hb*, were upregulated after cultivation in ARD soil with fold changes of 5.3 and 6.1, respectively.

Three genes upregulated in roots of all four genotypes after cultivation in ARD soils seem to be involved in regulating the molecular response to pathogen attack and/or plant defense. The chitinase B gene *CHIB* showed a significant fold change value of 14.6 in all root samples of plants grown in ARD soils. The gene

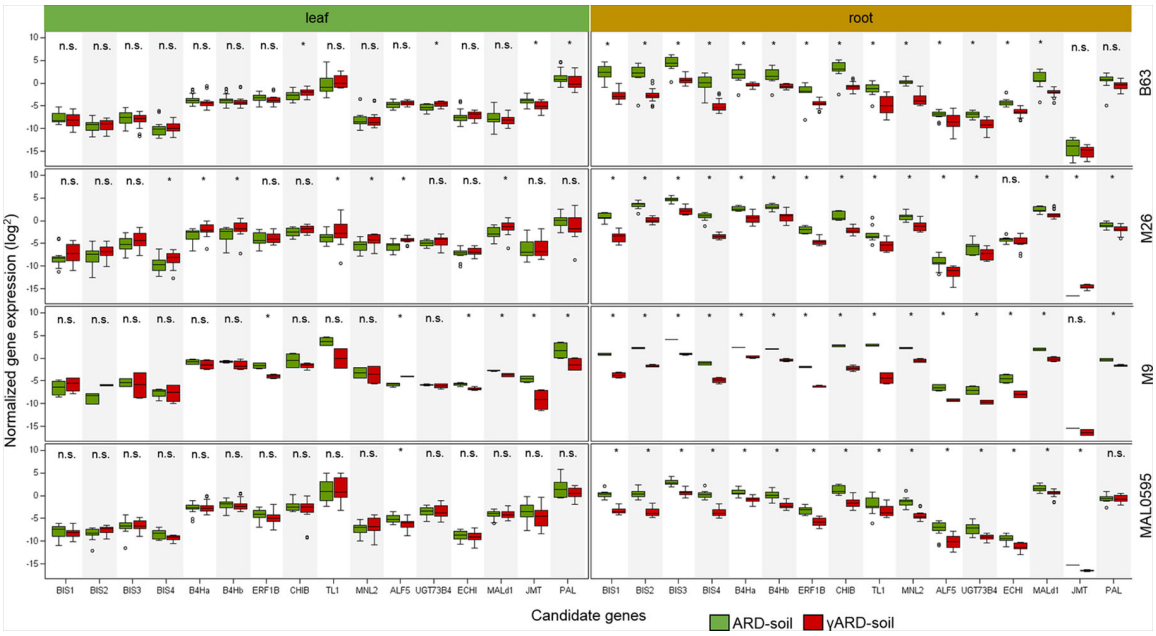

FIGURE 2 | Normalized gene expression values in leaf or root tissue of 16 highly upregulated candidate genes (CGs) in four different apple genotypes (B63, M26, M9 and MAL0595) grown in ARD and γARD soil, respectively. The normalized gene expression values are average values for the both soil origins (Meckenheim and Heidgraben). Non-significant values are indicated as n.s.; significant values ($p < 0.05$) are indicated with *. *BIS1*, biphenyl synthase 1; *BIS2*, biphenyl synthase 2, *BIS3*, biphenyl synthase 3; *BIS4*, biphenyl synthase 4; *B4Ha*, biphenyl 4-hydroxylase isoform a; *B4Hb*, biphenyl 4-hydroxylase isoform b; *ERF1b*, ethylene-responsive transcription factor 1B-like; *CHIB,* endochitinase EP3-like; *TL1*, thaumatin-like protein 1a; *MNL2*, putative mannosidase; *ALF5*, multi antimicrobial extrusion protein; *UGT73B4*, uridine diphosphate (UDP)-glycosyltransferase 73B4; *ECHI*, chitin-binding type 1; *Mal d1*, major allergen Mal d1-like; *JMT*, jasmonate O-methyltransferase-like; *PAL*, phenylalanine ammonia-lyase.

TL1 encoding the thaumatin-like protein was also upregulated in root samples with a significant fold change value of 19.4. The putative mannosidase gene *MNL2* being involved in cyanogenesis and defense response was upregulated with an average fold change of 11.3. For the ethylene-responsive transcription factor 1B-like (*ERF1B*), a fold change value of 8.5 was detected.

Within the multi antimicrobial extrusion protein family, an average fold change of 7.5 was detected in root samples for the gene *ALF5*. A significant upregulation (fold change 5.0, $p < 0.05$) was also detected for the chitin-binding type 1 gene (*ECHI*), which belongs to the endochitinase-like superfamily. Within the multigene family of plant glycosyltransferases, the uridine diphosphate (UDP)-glycosyltransferase (UGT) 73B4 encoding gene *UGT73B4* showed a 5.2-times higher expression in root tissue in ARD soils. The gene *Mal d1* encoding the major allergen Mal d1 showed an increased expression in root tissue with a significant fold change of 4.7.

The genes for jasmonate O-methyltransferase-like (*JMT*) and phenylalanine ammonia-lyase (*PAL*) were more strongly upregulated in leaves than in roots. For *PAL*, a fold change of 5.0 was detected in leaf tissue, whereas a fold change of 2.1 was found in roots (**Figure 3**). Fold changes for *JMT* were 4.8 in leaf tissue and 3.6 in roots. However, the difference in the expression level between samples from ARD soil and γARD soil was not significant for roots ($p = 0.21$) due to higher variability.

Expression of CGs in Response to Different Soil Origins

The 14 CGs with a significantly increased expression in roots were compared in plants grown in Meckenheim soil and in Heidgraben soil. As no data for M9 were available for Meckenheim soil, only B63, M26, and MAL0595 were considered for this comparison. Although differences in gene expression between the two soil types were found for all genes with a stronger upregulation in the soil from Meckenheim, the overall differences (including data of all genotypes) were mostly not statistically significant. The only exception is *MNL2*. This gene was expressed at a significantly higher level (3.2 times) in Meckenheim soil (18.4-fold to 5.7-fold, $p < 0.05$) (**Figure 4**).

Genotypic Differences in CG Expression

Genotypic differences in the expression of the 14 CGs were studied for all four genotypes after cultivation of plants in Heidgraben soil (**Figure 5**). *BIS1, BIS2, BIS3*, and *BIS4* were upregulated in all genotypes, except *BIS3* in MAL0595. The highest increase was found in B63, the lowest in MAL0595. The differences between these two genotypes were statistically significant. For *BIS2* and *BIS4*, significant differences were found

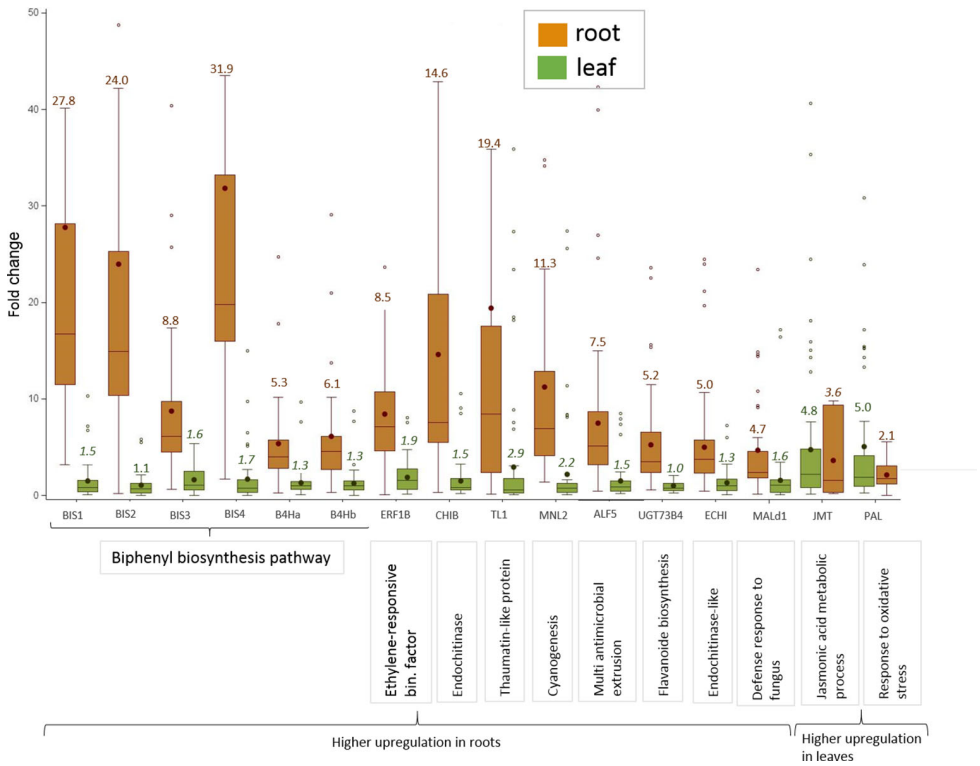

FIGURE 3 | Highly upregulated CGs in apple grown in ARD soils with an average ΔΔCq-fold change value > 4.5 in either root or leaf tissue after cultivation in ARD soil and their assignment to molecular function. The fold change values (ARD soils/γARD soils) are average values for the four genotypes, including five (M26, B63 and MAL0595) or two replicates ("M9"), respectively and the two soils (Meckenheim and Heidgraben). Non-significant fold change values are indicated in italics. The whiskers were not drawn to the minimum or maximum values, if they were longer than 1.5 times the interquartile range (IQR). Data points outside of this range of 1.5 × IQR were indicated as outliers (dots).

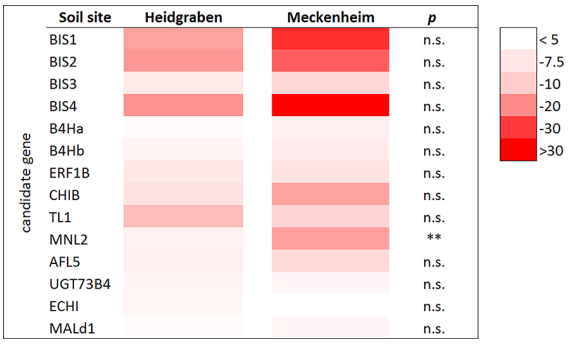

FIGURE 4 | Regulation of CG expression in roots of plants grown in the ARD soils Heidgraben and Meckenheim including mean gene expression data of the genotypes B63, M26, and MAL0595, with five replicates for each genotype. The heat map indicates the fold change values (ARD soil/γARD soil) with the rows displaying the selected 14 CGs with an average significant fold change value > 4.5 in root tissue. The columns display the soil sites Heidgraben and Meckenheim. The intensity of the red color corresponds with the detected fold change value. $p < 0.01$ (**) and n.s., not significant.

between B63 and the three genotypes M26, M9, and MAL0595. The upregulation of *BIS1* and *BIS3* was comparable for B63, M9, and M26, but significantly lower in MAL0595. The least differences were found for *B4Ha* and *B4Hb*. Significant differences were detected only between B63 and M26, for *B4Ha*.

MNL2 showed the highest upregulation in B63 and the lowest in MAL0595. Statistically significant differences were also detected between B63/M9 and M26,/MAL0595. Genotypic differences were also found for *CHIB, ERF1B*, and *TL1*. For *ERF1B*, the fold changes were highest in M9 and lowest in M26,. For *CHIB* and *TL1*, the fold changes were highest in M9 and lowest in MAL0595. Differences were statistically significant between M9 and the other genotypes (**Figure 5**).

For *ECHI,* statistically significant differences were found between M26, (lowest regulation) and M9 (highest regulation). *Mal d1* showed highest upregulation in B63 with statistically significant differences to M26, M9, and MAL0595. The fold change of *UGT73B4* was less pronounced. Nevertheless, the detected differences were statistically significant between M26, and MAL0595 and between M26, and M9. No genotype-specific differences were found for *AFL5*.

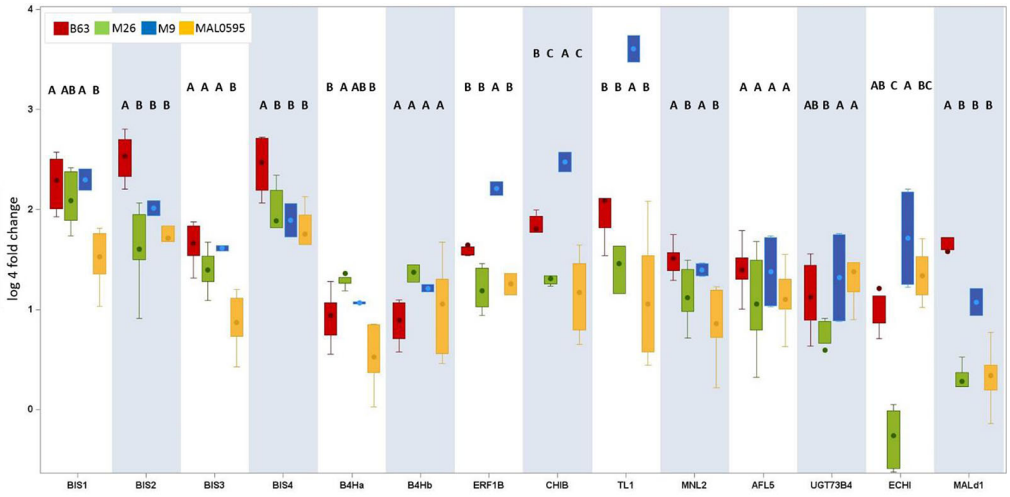

FIGURE 5 | Genotypic differences in the regulation of the 14 CGs. This box plot presents only CGs with an upregulation (fold change ARD soil/γARD soil > 4.5) in root tissue. The root-specific average fold change values of all genotypes including five (B63, M26, MAL0595) or two replicates (M9), respectively, of 14 genes after cultivation in Heidgraben ARD soil are shown. The letters denote the significant differences between the genotypes for one gene. Significant differences are indicated by different letters. The whiskers were not drawn to the minimum or maximum values, if they were longer than 1.5 times the IQR. Data points outside of this range of 1.5 × IQR were indicated as outliers (dots).

Phytoalexin Biosynthesis in Roots

A total of 12 biphenyl and dibenzofuran phytoalexins were detected and quantified in the roots of the *Malus* rootstock genotypes M26, B63, and MAL0595, which were grown in the two different soils for 4 weeks (**Figure 6A**). Significant differences in phytoalexin production were observed among the genotypes. M26, roots contained the highest phytoalexin amount and MAL0595 had the lowest, while B63 had an intermediate level of phytoalexins (**Figure 6B**). Furthermore, MAL0595 formed only three biphenyls and two dibenzofurans, whereas M26 and B63 produced the majority of the four biphenyls and eight dibenzofurans analyzed (**Table S1**). Notably, phytoalexin biosynthesis was significantly induced by ARD soils from both sites, whereas the difference in total phytoalexin content between the two soil sites was not significant (**Figure 6C**). Among the five main phytoalexins detected, the amount of 2-hydroxy-4-methoxydibenzofuran with a retention index (RI) of 2,131 was the highest. It was the only compound that was observed in all the samples including those from γARD soils. The content of 2-hydroxy-4-methoxydibenzofuran was upregulated by the ARD soils in all genotypes. The same held true for the other four major phytoalexins (RI 2,090; 2,121; 2,228, and 2259, respectively), except for aucuparin (RI 2,090), which was downregulated in MAL0595 in both soil types (**Table S1**). Another phytoalexin, 2'-hydroxyaucuparin (RI 2,193), also showed an interesting soil-dependent regulation pattern. In Heidgraben ARD soil, its content was upregulated in all genotypes; however, in Meckenheim ARD soil its content was downregulated (**Table S1**). However, differences between the three genotypes in their responses to the two soil sites were also observed. In MAL0595 roots, the formation of new phytoalexin compounds was not induced by ARD soil from both sites, while the formation of five and eight new phytoalexins was induced by ARD soil in M26, and B63 roots, respectively. Furthermore, M26 and B63 formed only two and four phytoalexin compounds, respectively, when grown in Heidgraben γARD soil, but produced eight phytoalexin compounds each in Meckenheim γARD soil (**Figure S1**).

Correlation Between Phenotypic Data, Gene Expression Data, and Phytoalexin Contents

A Pearson's correlation was calculated between gene expression (fold changes of the 14 CGs expressed in root tissue) and changes in biomass and shoot length. The highest correlation was found between *CHIB* expression and shoot length ($r = 0.96$; $p < 0.01$). Highly significant correlations were also found between *B4Ha, BIS1, BIS3,* and *BIS4* expression and shoot length (**Table 3**). A statistically significant correlation between the biomass and the expression of any of the CGs was not observed.

Statistically significant correlations were also found between some phytoalexin compounds and the changes in expression of the six CGs belonging to the biphenyl biosynthesis pathway. The amount of noraucuparin was most strongly correlated with the expression of *B4Ha*, *BIS1*, *BIS3*, and *BIS4* ($r = 0.70$ to 0.73; $p = 0.01$, **Table 4**). *B4Ha* expression was correlated with the amount of 2-hydroxy-4-methoxydibenzofuran and the isomer of noraucuparin. A significant correlation was found between the total amount of phytoalexins and the changes in expression of *B4Ha* ($r = 0.60$; $p = 0.04$).

Protein–Protein Interaction Analysis

Accession numbers of 17 DEGs with a significant fold change > 1.5 in roots were integrated into a protein interaction network

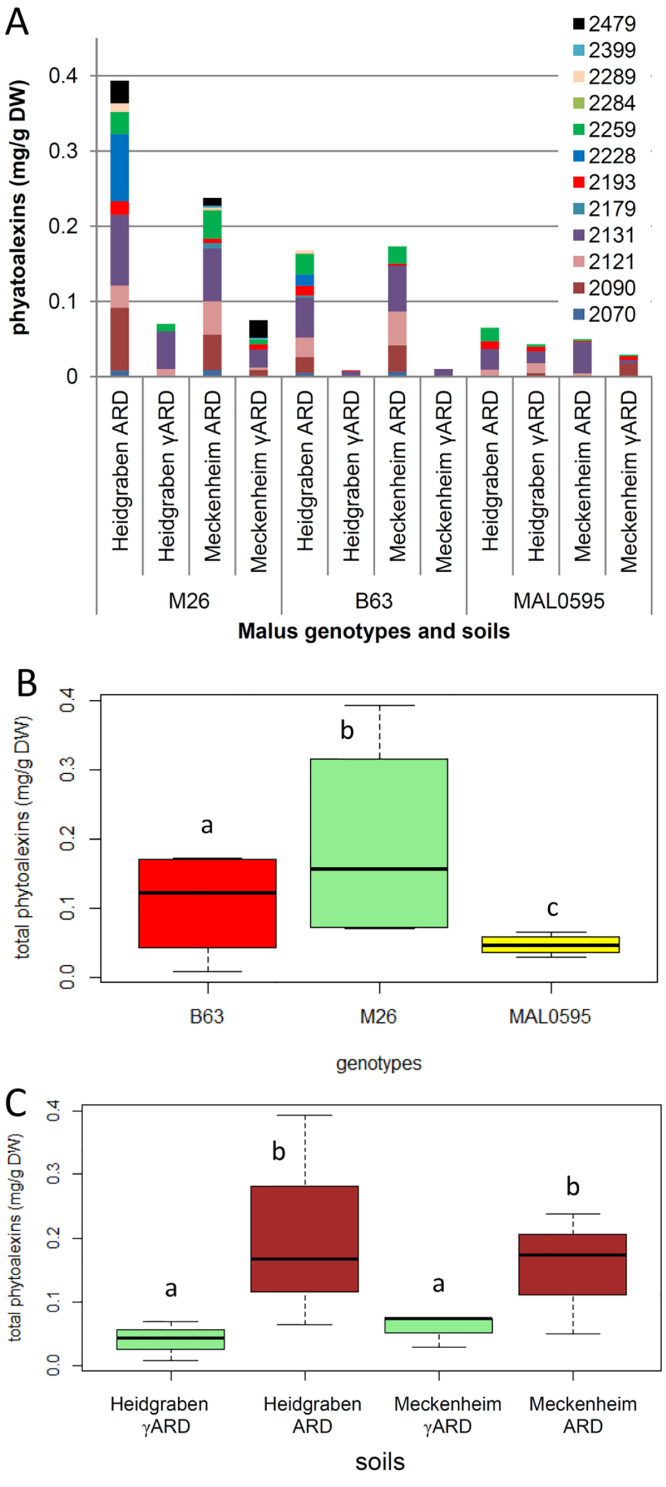

FIGURE 6 | Analysis of phytoalexins in roots of the three genotypes M26, B63, and MAL0595, which were grown for 4 weeks on ARD and γARD soils from the two sites Heidgraben and Meckenheim. **(A)** Levels of individual phytoalexins identified by gas chromatography–mass spectrometry (GC-MS). Compound retention index (RI) 2,070, isomer of noraucuparin; 2,090, aucuparin; 2,121, noraucuparin; 2,131, 2-hydroxy-4-methoxydibenzofuran; 2,179, isomer of eribofuran; 2,193, 2'-hydroxyaucuparin; 2,228, eribofuran; 2,259, norerioboﬀuran; 2,284, isomer of hydroxyeribofuran; 2,289, isomer of norerioboﬀuran; 2,399, methoxyeribofuran; 2,479, 3,9-dimethoxy-2,4-dihydroxydibenzofuran. **(B, C)** Total phytoalexin content as a function of genotype and soil, respectively. Different letters indicate significant differences revealed by Tukey test (n = 8 for B and n = 6 for C) applied to the total phytoalexins.

TABLE 3 | Pearson's correlation between the differences in the expression of the 14 candidate genes (CGs, expressed in fold changes) and the differences in fresh biomass and shoot length of plants grown in ARD soil compared to those grown in γARD soil (biomass/ shoot length in ARD soil - biomass/ shoot length in γARD soil), significant correlations are given in bold with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

CG	Biomass[1]	Shoot length[1]
BIS1	–0.57	**–0.92****
BIS2	–0.26	–0.65
BIS3	–0.46	**–0.86***
BIS4	–0.57	**–0.91****
B4Ha	–0.41	**–0.82***
B4Hb	–0.31	–0.77
ERF1B	–0.38	–0.49
CHIB	–0.67	**–0.96*****
TL1	–0.23	–0.15
MNL2	–0.11	–0.49
AFL5	0.21	0.39
UGT73B4	–0.44	–0.21
ECHI	0.01	0.11
Mal d1	–0.44	–0.69

[1]*Measured after 28 days of cultivation in the greenhouse.*

using TAIR (The Arabidopsis Information Resource, 6). These proteins included the highly expressed *BIS* (fold change > 20.0), *CHIB* and *MNL2* (fold change > 10.0), as well as *ERF1B, B4H*, and *PAL* (fold change > 4.5). For the remaining 22 DEGs, no interaction was found.

The highest confidence of a protein–protein association was found in the first network cluster (**Figure 7**). This cluster consisted of *BIS*, *CHIA*, *O*-methyltransferase 1 (*OMT1*), polyphenol oxidase (*PPO*), *PAL*, anthocyanidin reductase (*ANR*), and anthocyanidin synthase (*ANS*). Two further proteins within this cluster are involved in the signal transduction process (*CHIB*) and the oxidation–reduction process (flavanone 3-hydroxylase, *FLS*). *BIS* showed the highest confidence of interaction with *ANR, ANS,* and *FLS*. The confidence of interaction of *BIS* with other proteins within this cluster was medium to high.

The second cluster was comprised of four proteins with a medium to high confidence of interaction. Three proteins (*ERF1B*, transcription factor *MYC2,* pathogenesis-related protein *PR-4*) were involved in the signaling pathway, whereas one seems to be involved in abscisic acid biosynthesis (nine-*cis*-epoxycarotenoid dioxygenase 3, *NCED3*).

The third cluster contained four proteins of different functions. These proteins were grouped with a low to medium confidence of interaction. *B4H* is involved in phytoalexin biosynthesis, whereas tetracetide *alpha*-pyrone reductase 1 (*TKPR1*) belongs to the brassinosteroid biosynthesis pathway. Indole-3-acetic acid-amido synthetase (*GH3.1*) belongs to the auxin biosynthesis pathway. The function of *MNL2* is unknown, but it is associated to the oxidoreductase family.

DISCUSSION

Phytoalexin Biosynthesis Is Strongly Increased in Response to ARD

It is generally accepted that ARD is strongly associated with an unbalanced complex of soil biota, including bacteria, fungi, oomycetes, and nematodes (Rumberger et al., 2007; Kanfra et al., 2018). In the present study, the expression changes of 108 ARD CGs were evaluated in roots of three different *Malus* rootstocks and one wild apple genotype grown in ARD soils from two different sites in Germany. The most highly upregulated CGs in ARD soil were genes related to the phytoalexin biosynthesis, including the four biphenyl synthase genes *BIS1*, *BIS2*, *BIS3*, and *BIS4* and the two biphenyl 4-hydroxylase genes *B4Ha* and *B4Hb* (**Figure 4**). *BIS* and *B4H* genes encode for enzymes involved in the biosynthesis of biphenyl and dibenzofuran phytoalexins (**Figure S2**). These phytoalexins are only formed by plants belonging to the subtribe Malinae of the family Rosaceae, such as members of the genera *Malus* and *Pyrus* (Liu et al., 2007; Beerhues and Liu, 2009; Liu et al., 2011; Chizzali and Beerhues, 2012; Sircar et al., 2015). The results of CG expression correlated well with the total phytoalexin content, which was also significantly increased in the roots of plants grown in ARD soils (**Table 4**, **Figure 5C**). It has to be mentioned here, that the roots were sampled 3 weeks earlier for gene expression analyses than for phytoalexin detection, because after 1 week of culture, the amount of root fresh mass was not sufficient to enable both kinds of analyses. Moreover, the culturing period of 4 weeks was necessary to record the biomass data that allowed a clear classification of the soils as ARD soils based on the observed growth depression.

Comparable results for the expression of these CGs genes were also obtained in other studies on apple, either in response to the necrotrophic pathogen *P. ultinum* or in response to ARD soil

TABLE 4 | Pearson's correlation between changes in the expression of the six CGs of the phytoalexin biosynthesis pathway (expressed as fold changes) and the amounts of individual phytoalexins, $p < 0.05$ (*) and $p < 0.01$ (**). Intensity of red shading visualizes strength of correlation. Significant correlations are given in bold.

Phytoalexins	Candidate gene					
	BIS1	BIS2	BIS3	BIS4	B4Ha	B4Hb
2-hydroxy-4-methoxydibenzofuran	0.52	0.45	0.54	0.51	**0.65** *	**0.58**
Aucuparin	0.40	0.20	0.41	0.33	0.56	0.47
Isomer of noraucuparin	**0.57**	0.41	**0.57**	0.54	**0.64** *	0.54
Noraucuparin	**0.73** **	0.55	**0.71** **	**0.70** **	**0.73** **	0.67
Norerioboforan	0.49	0.40	0.51	0.49	0.52	0.49
Phytoalexins total	0.44	0.29	0.46	0.38	0.60 *	0.53

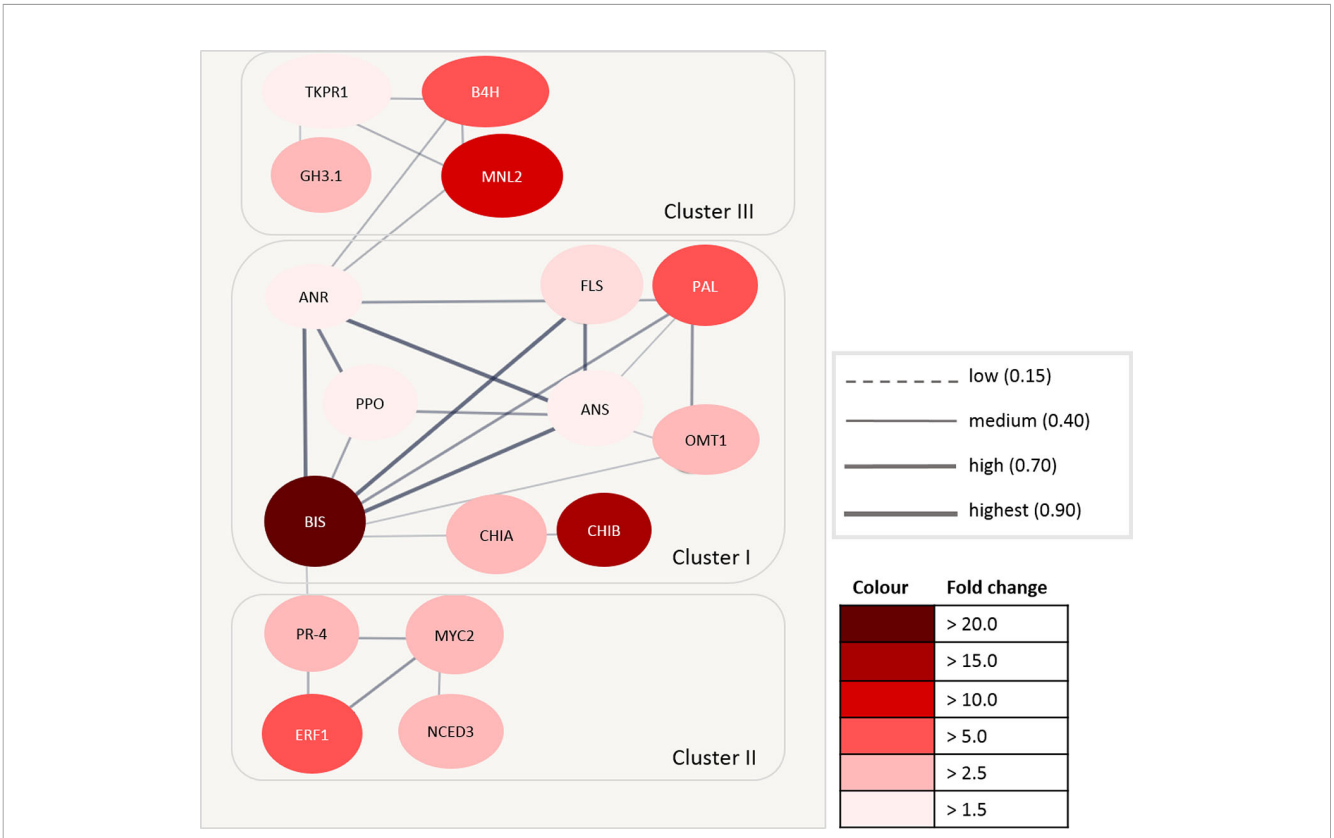

FIGURE 7 | Protein–protein interaction matrix using The Arabidopsis Information Resource (TAIR) accession number of differentially expressed genes (DEGs) in *Malus*. The circles represent the proteins, and the lines between circles represent the interaction between individual proteins. The thickness of the lines defines the confidence of the interaction. The intensity of the red color indicates the fold change expression value (ARD soil/γARD soil). *ANR*, anthocyanidin reductase; *ANS*, anthocyanidin synthase; *B4H*, biphenyl 4-hydroxylase; *BIS*, biphenyl synthase; *CHIA*, acidic endochitinase-like; *CHIB*, endochitinase EP3-like; *ERF1*, ethylene-responsive transcription factor 1; *FLS*, flavanone 3-hydroxylase; *GH3.1*, indole-3-acetic acid-amido synthetase; *MNL2*, putative mannosidase; *NCED3*, nine-cis-epoxycarotenoid dioxygenase 3; *TKPR1*, tetraketide alpha-pyrone reductase 1; *MYC2*, transcription factor MYC2; *OMT1*, O-methyltransferase 1; *PAL*, phenylalanine ammonia-lyase; *PPO*, polyphenol oxidase; *PR-4*, pathogenesis-related protein PR-4.

(Zhu et al., 2014; Weiß et al., 2017a; Weiß et al., 2017b; Zhu et al., 2017). Phytoalexins are part of the complex defense system of plants against pests and pathogens (Jeandet et al., 2014). The induction of phytoalexin biosynthesis seems to be one of the induced defense responses of *Malus* rootstocks to stresses caused by the biota in ARD soils. The antifungal and antibacterial activities of biphenyls and dibenzofurans was clearly shown although their precise mechanisms of action are still unknown (Chizzali and Beerhues, 2012). Loss-of-function experiments on other plant–pathogen interactions have demonstrated that reduced levels of phytoalexins lead to increased disease susceptibility (Jeandet et al., 2014). Examples are known from pea (Wu and VanEtten, 2004), soybean (Graham et al., 2007), sorghum (Ibraheem et al., 2010), pear (Chizzali et al., 2016), and *Arabidopsis* (Jeandet et al., 2013). However, there are also reports that high phytoalexin concentrations may be toxic to plant cells (Dixon et al., 1994; Rogers et al., 1996), which was also hypothesized by Weiß et al. (2017b) for apple rootstocks. The accumulation of high concentrations of phytoalexins in ARD-susceptible rootstocks like M26 and B63 may cause root damage and even death. It was assumed that the exudation mechanism or the detoxification system do not work properly in these genotypes. This hypothesis is supported by the results obtained with the less susceptible genotype MAL0595. This genotype accumulated significantly less phytoalexins in roots compared to M26 and B63. Consistently, the reduction in shoot length of MAL0595 plants grown in ARD soils was not statistically significant (**Figure 6C**, **Table 2**).

Among the four *BIS* genes, the highest upregulation in ARD soils was observed for *BIS1*, followed by *BIS2* and *BIS4*. However, *BIS3* transcript level exceeded the transcript levels of the other *BIS* genes in the roots by far (**Table S5**). As previously reported by Chizzali et al. (2012a) and other authors, the regulation of the individual *BIS* genes can differ depending on the pathogen and the type of the infected tissue. In a transcriptome analysis conducted with M26 grown in ARD soil, the expression of *BIS2*, *BIS3*, and *BIS4* was induced, with *BIS3* showing the highest increase in roots (Weiß et al., 2017a). In the present study, *BIS3* expressional levels also were the highest (**Table S5**) but with the lowest fold change among the *BIS* genes investigated

in the roots. Due to its exceedingly high expression level, *BIS3* seems to play a pronounced role in phytoalexin biosynthesis. *BIS4* showed the highest differences between the two soil types. After fire blight infection, *BIS1* and *BIS2* were upregulated in leaf tissue. In contrast, *BIS3* was strongly expressed in the stem, where it was spatially limited to the transition zone between healthy and necrotic tissue (Chizzali et al., 2016). In the present study, expression of *BIS* genes was also focused on the region affected by the biotic stress, the roots.

Further CGs Involved in Biotic Stress Responses Are Upregulated

Primer efficiencies were calculated in a different experiment with a different PCR system for validation. In the Fluidigm system, the software calculates a quality score for each individual amplification curve by comparing the amplification curve to an ideal exponential curve. If the curve is close to the ideal one, the quality score approaches 1. The software sets a cutoff for the quality score of > 0.65 to exclude primers with poor efficiencies. Nevertheless, all data used were still without any PCR efficiency correction. Therefore, we decided not to consider smaller differences in gene expression, but focused on the CGs with fold changes above 4.5.

Among these, *TL1* and *Mal d1* were upregulated in roots after cultivation of plants in ARD soil. Similar results were obtained by Weiß et al. (2017a). The *TL1* product belongs to a highly complex protein family with antimicrobial and antifungal activities (Liu et al., 2010; Singh et al., 2013). Overexpression of *TL*s in transgenic wheat plants mediated enhanced resistance and protection against different fungal pathogens (Mackintosh et al., 2007). Mal d1 is a defense protein, which belongs to group 10 of pathogenesis-related proteins. It is expressed by plants in response to different stress conditions, such as pathogen infection, exposure to certain chemicals, wounding, and stressful environmental conditions (Puehringer, 2003). In apple fruits, Mal d1 is known as a birch pollen–related food allergen. Previous studies by our research group have shown that the synthesis is strongly related to exogenous stress factors (Schmitz-Eiberger and Matthes, 2011; Kiewning and Schmitz-Eiberger, 2013). However, its function in response to ARD remains to be elucidated.

ERF1B, *CHIB*, and *ECHI* also showed a notable fold change in root samples. *ERF1B* encodes a transcription factor that is involved in ethylene signaling. An *ERF1B*-mediated ARD defense response in apple roots was also observed in other studies (Shin et al., 2014; Weiß et al., 2017a). Ethylene is an essential mediator of biotic and abiotic stress responses (Müller and Munné-Bosch, 2015), and ethylene-responsive transcription factors (*ERF*) regulate the molecular response to pathogen attack (Ito et al., 2014; Müller and Munné-Bosch, 2015; Huang et al., 2016). Within the ethylene-mediated transcriptional response, the promoter region of *CHIB* may be a target of *ERF* transcription factors. Based on the results obtained, it could be assumed that the changes in *ERF* expression have led to a subsequent activation of *CHIB* (Shin et al., 2014). For other genes like *ACS* and *ACO*, which encode key enzymes of the ethylene biosynthesis, no upregulation was observed. It is common knowledge that different isoforms within a gene family can carry out specific functions in different plant processes (Shin et al., 2014). An involvement of other isoforms of *ACS* and *ACO*, which were not investigated in this study, cannot be excluded. The endochitinase EP3-like gene *CHIB* belongs to a large family of plant chitinase genes and is generally induced by pathogen attack and other biotic stresses (Hamid et al., 2013; Nagpure et al., 2014). Chitinases play a role in the biocontrol of fungal phytopathogens and plant defense systems especially against chitin-containing pathogens (Hamid et al., 2013).

The genes *MNL2, ALF5, ECHI*, and *UGT73B4* were also significantly upregulated in roots. These genes appear to be related to ARD, but have not been described in this context before. The putative mannosidase gene *MNL2* belongs to the glucose–methanol–choline oxidoreductase family. Genes of this family are involved in adaptive processes in plant–insect interactions during host-dependent chemical defense (Rahfeld et al., 2014). However, the detailed function of the *MNL2* gene in plants is still unknown. The *ALF5* gene belonging to the MATE gene family is expressed in root epidermis cells and is necessary for protecting roots from toxic compounds in the soil (Diener et al., 2001). Some genes within the MATE gene family are supposedly involved in transporting toxic compounds to infected parts of the plant in order to attenuate pathogen attack (Santos et al., 2017). Within the multigene family of plant UGTs, an upregulation was observed for *UGT73B4*. Plant glycosyltransferases usually use UDP-glucose in the transfer reactions catalyzed. Furthermore, it is assumed that UGTs are part of stress responses (Li et al., 2001; Langlois-Meurinne et al., 2005). Analysis of *A. thaliana* defense-signaling mutants indicated that expression of the corresponding UGT genes is necessary during the hypersensitive response (Dare et al., 2017). These results emphasize the importance of UGTs in plant–pathogen interactions (Dare et al., 2017). It is tempting to speculate that UGTs may be involved in the detoxification of biphenyl and dibenzofuran phytoalexins via glycosylation and deposition in the central vacuole. However, no glycosylated derivatives of the defense compounds have so far been detected in infected plants and elicitor-treated cell cultures of the Malinae, except for two glucosides (aucuparin and eriobofuran derivatives), which were isolated from cell cultures of the scab-resistant apple cultivar 'Liberty' (Borejsza-Wysocki et al., 1999). Because of their general function in pathogen defense, *ALF5*, *ECHI*, and *UGT73B4* appear to also be activated by pathogens of the ARD complex. However, further investigations will be necessary to elucidate their precise function.

Two CGs Showed Upregulation in Leaf Tissues

PAL and *JMT* showed a stronger upregulation in leaf tissue than in roots. The *PAL* gene encodes for the enzyme phenylalanine ammonia-lyase, which is the key enzyme of the phenylpropanoid pathway. Repression of this pathway in apple via a reduction in key transcript levels (e.g. for *PAL*), and enzyme activities (e.g.

PAL and chalcone synthase) resulted in severe dwarfing and internode length reduction (Dare et al., 2017). The occurrence of stunted shoots because of ARD infection seems therefore to be independent of the *PAL* gene expression level. Whether shoot stunting is connected to the occurrence or the amount of individual phenolic compounds or not remains to be investigated. The *JMT* gene encodes for the enzyme S-adenosyl-l-methionine:jasmonic acid carboxyl methyltransferase (*JMT*), which catalyzes the formation of methyl jasmonate from jasmonic acid. Plants produce jasmonic acid and methyl jasmonate in response to many biotic and abiotic stresses, in particular, herbivory and wounding (Seo et al., 2001; Wasternack, 2007). Both genes (*PAL* and *JMT*) are associated with pathogen defense reactions and stress response. The upregulation of their expression in leaf tissue could be an indication for biotic stress because of ARD infection. However, their precise role in connection with ARD has to be further investigated.

The Soil Origin Influences Plant Growth, CG Expression and Phytoalexin Production

The expression of CGs was compared between plants grown in Meckenheim soil and Heidgraben soil. The genes *MNL2, BIS1, BIS2*, *BIS4*, and *TL1* showed a strong upregulation in roots of all genotypes if plants were grown in ARD soil. This was the case for both soil types, although the upregulation was more pronounced in plants grown in Meckenheim soil (**Figure 4**). Even though a stronger increase in gene expression was observed, the total phytoalexin amount was not increased in plants of Meckenheim soil. The differences found between the two soils were not statistically significant (**Figure 6C**). It is assumed that each genotype seems to produce phytoalexins up to a certain level, once the biosynthesis is stimulated by ARD soil. So far, the rate-limiting steps of biphenyl and dibenzofuran biosynthesis remain unknown. Compared to the *BIS* genes, the fold changes in the expression of *B4Ha* and *B4Hb* were markedly lower, and genes for *O*-methyltransferases (Khalil et al., 2015) were not among the upregulated genes. Due to the incomplete examination of the phytoalexin biosynthetic pathway, some genes remain to be identified, including the gene coding for the enzyme that converts aucuparin to 2'-hydroxyaucuparin (**Figure S2**). This gene should be highly expressed in ARD soil from Heidgraben, because the 2'-hydroxyaucuparin content of roots grown in this soil was greatly increased compared to that of roots from γARD soil. In roots of plants grown in ARD soil from Meckenheim, the reaction of this gene might be different; the expression of this gene does not seem not to be induced or even inhibited because of the decrease of the 2'-hydroxyaucuparin content in samples of these roots (**Figure S1**). Thus, different ARD soils may differently affect individual phytoalexin biosynthetic steps, leading to qualitative and/or quantitative changes in the phytoalexin patterns. Previously, varying phytoalexin patterns were observed in cell cultures of *Sorbus aucuparia* upon treatment with different elicitors, which, for example, stimulated the accumulation of aucuparin or eriobofuran as the major components (Hüttner et al., 2010). In the present study and a previous one (Weiß et al. 2017b), relatively high levels of 2-hydroxy-4-methoxydibenzofuran were even detected in roots from γARD soils. This indicates that the formation of this compound does not necessarily need the ARD biome stimulus although ARD soils lead to a further strong increase in the accumulation.

Soil properties can influence the extent of ARD directly or indirectly (von Bronsart, 1949; Franke-Whittle et al., 2018; Mahnkopp et al., 2018). The soil pH value is one of these properties, which has been discussed several times in this context. However, the results published about the effect of the soil pH value onto ARD severity were contrasting. In some cases, it was shown that a low soil pH seemed to be associated with a high degree of ARD (Willett et al., 1994; Mahnkopp et al., 2018). In other studies, it was found that ARD symptoms were less pronounced in soils with a low pH value (Jonkers et al., 1980; Utkhede et al., 1992). We found a higher overall fold change of CG expression on the silty soil of Meckenheim with a high pH (6.7) compared to the sandy soil (Heidgraben) with a lower pH value of 5.3. However, the effect of soil pH on ARD should not be overestimated. Changes in pH are not induced by apple replanting and are generally an unstable parameter (Mahnkopp et al., 2018). Different rootstocks seem to have a different growth optimum regarding the soil pH value. Some rootstocks achieve optimal growth at a low pH (e.g. CG.6589), whereas other rootstocks are well adapted to more calceous soils (e.g. CG41) (Fazio et al., 2012).

Soil organic matter (SOM) seems also to reduce the induction of ARD (Franke-Whittle et al., 2018). In our study, a remarkably lower SOC (soil organic carbon = total carbon due to absence of carbonate) of 12.3 g kg^{-1} in Meckenheim soil compared to a SOC of 25.4 g kg^{-1} in Heidgraben soil (**Table 1**), corresponded to a higher CG regulation. Plant growth in terms of shoot and root fresh biomass was significantly reduced on both soils for the sensitive genotypes M26, and B63 (**Table 2**). The only exception was root fresh mass of B63, which was not significantly reduced when grown on Meckenheim soil. These observations are interesting as they stress the limits of growth-based bio-tests to determine ARD severity. Factors like the high available water capacity of the loamy Meckenheim soil in comparison to the sandy Heidgraben soil may explain differences in plant growth besides ARD severity.

Soil biota like nematodes, which are part of the ARD complex, are also influenced by soil texture (Hbirkou et al., 2011). Is has been described that sandy soils are often more prone to ARD than loamy soils (Mahnkopp et al., 2018; Winkelmann et al., 2019). In this study, the upregulation of the CGs was less pronounced in the sandy Heidgraben soil compared to the silty Meckenheim soil, which is not in agreement with the findings mentioned above. Similar observations were made by Fazio et al. (2012) in a study investigating the influence of soil pH value and soil texture on ARD. Based on plant growth, some rootstocks appeared to be less sensitive to ARD in clay soil than in sandy soil, but also the opposite relationship was observed because other rootstock cultivars appeared to be more sensitive to ARD in the clay soil compared to the sandy soil (Fazio et al., 2012).

These results indicate that soil properties cannot be judged without knowledge of the soil biome composition. Likewise, Mazzola and Manici (2012) concluded that abiotic factors may reduce or intensify ARD, but up to now, a causal relationship of a single abiotic factor and ARD is not evident.

Many soil characteristics influence plant growth. Therefore, growth-based bio-tests are limited in their information value regarding ARD severity. Additional methods for a more reliable diagnosis and possible quantification of ARD are of interest. Our data suggest that the expression of certain CGs may be a starting point for the identification of early indicators as an addition to growth data. To evaluate their usefulness in different ARD situations, especially under field conditions, further studies are necessary. These studies should also include the comparison to virgin soils on which no Rosaceae plants had been grown before and which are collected close to the replant sites.

Genotypic Differences are Found for Gene Expression in Response to ARD

The four *Malus* genotypes M9, M26, B63, and MAL0595 used in our gene expression study possess susceptibility to ARD to different extents. M9 and M26, were previously classified as susceptible genotypes (Isutsa and Merwin, 2000; Leinfelder and Merwin, 2006; St Laurent et al., 2010). MAL0595 was grouped as a less sensitive genotype (Cummins and Aldwinckle, 1983; Reim et al., 2019). By contrast, no information regarding its ARD reaction was available for the rootstock genotype B63 at the beginning of our experiment. Recently, root microscopic and bio-test data proved this genotype to react similarly to M26, in response to ARD (Grunewaldt-Stöcker et al., 2019). Our present results on plant growth and CG expression support the observation that B63 has to be considered as ARD-sensitive.

In the present study, the genotype-specific gene expression data (**Figure 5**) are consistent with the phytoalexin detection results (**Figure 6B**). Relatively low gene expression levels yield a relatively low total phytoalexin content in MAL0595, whereas high gene expression levels lead to high phytoalexin contents in M26, and B63 (**Figures 4** and **5C**). Besides the quantitative differences, there was also qualitative variation in the phytoalexin patterns. In γARD soils, roots of MAL0595 formed few phytoalexins at low levels, which increased partly upon growth in ARD soils from the two sites. While the 2-hydroxy-4-methoxydibenzofuran content was strongly enhanced, the noraucuparin content decreased (**Figure S1**). No new phytoalexins were formed. In M26, and B63, the aucuparin, noraucuparin, and noreriobofuran levels increased greatly in both ARD soils. Therefore, these three phytoalexins may be the major compounds that cause cytotoxicity to apple roots. For the growth data, the results for MAL0595 were opposite to those for M26, and B63. Thus, the low fold changes of most CGs in response to ARD (**Figure 6**), the low phytoalexin content compared to the other genotypes (**Figures 5** and **S1**), and the low growth depression (**Table 2**) match the classification by phenotypic data of MAL0595 as less susceptible to ARD. The assessment of these parameters may similarly provide information about the degree of ARD susceptibility of other genotypes. Further studies should clarify if the *BIS* genes can be used as possible biomarkers for ARD susceptibility, as their expression correlated well with the observed susceptibility as classified on phenotypic data. *BIS3* seems to play a key role in phytoalexin biosynthesis under replant conditions, because *BIS3* transcript levels exceeded the other *BIS* genes by far, but overall they were regulated less strongly.

CONCLUSIONS

The expression of 108 CGs was studied in leaf and root tissue of four different *Malus* genotypes grown in ARD soil and γARD soil from two different sites in Germany. For most of these genes, it is the first time that their tissue specific expression pattern was investigated in different genotypes and in response to ARD. The data obtained allow conclusions about general (genotype-independent) and genotype-dependent effects of ARD on the expression of these genes. Changes in CG expression were more frequent and more pronounced in root tissue compared to leaf tissue. This result suggests that the response of *Malus* plants to ARD is local. The defense reaction seems to be spatially restricted to the site of infection. A systemically acquired stress response could not be detected. Sixteen CGs were strongly upregulated in roots of plants grown in ARD soil. Six of them belong to the phytoalexin biosynthesis pathway. Their expression patterns were consistent with the phytoalexin content. It can be assumed that phytoalexins may play a role in the reaction of *Malus* plants to ARD. However, their function in the disease etiology remains to be clarified. The expression patterns of the biphenyl synthase genes *BIS1*, *BIS2 BIS3*, and *BIS4* correlated well with the phenotypic reaction of the *Malus* genotypes investigated with *BIS3* showing the strikingly highest normalized expression. These genes are useful as biomarkers to identify the presence of ARD inducing microbiota in unknown soil samples. They may also give clear indications for the defense reaction of plants growing at a site, whose state with regard to ARD is unknown.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135081. Data from the greenhouse experiment is deposited in the BonaRes Data Center (DOI: 10.20387/bonares-R1S0-52TX).

AUTHOR CONTRIBUTIONS

SR and A-DR were involved in performing the experiments, analyzing the data, and writing the manuscript. SW and MS contributed to CG selection and writing the manuscript. BL and LB conceived and designed the phytoalexin experiments. BL and A-DR analyzed the phytoalexin compounds in root material and interpreted the data. HF, M-VH, and TW conceived and

coordinated the project, and participated in data interpretation and writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01724/full#supplementary-material

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3 Identification and validation of early genetic biomarkers for apple replant disease

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RESEARCH ARTICLE

Identification and validation of early genetic biomarkers for apple replant disease

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Abstract

Apple replant disease (ARD) is a serious threat to producers of apple trees and fruits worldwide. The ARD etiology is not unraveled and managing options are either economically not applicable or environmentally harmful. Thus, interest is given in biomarkers that allow to indicate ARD situations at early time points in order to classify soils according to ARD severity but also to analyze the effectiveness to potential countermeasures. This study aimed at (i) identifying ARD biomarkers on the transcriptional level in root tissue by analyzing the expression of previously identified candidate genes in ARD soils of different origin and texture and (ii) testing the specificity of these marker genes to ARD. *In vitro* propagated M26 plantlets were submitted to a bio-test with three ARD soils, either untreated or disinfected by γ-irradiation. Expression of seven candidate genes identified in a previous transcriptomic study was investigated by RT-qPCR in a time course experiment. Already three days after planting, a prominent upregulation of the phytoalexin biosynthesis genes *biphenyl synthase 3 (BIS3)* and *biphenyl 4-hydroxylase (B4Hb)* was observed in the untreated ARD variants of all three soils. The phytoalexin composition in roots was comparable for all three soils and the total phytoalexin content correlated with the expression of *BIS3* and *B4Hb*. The third promising candidate gene that was upregulated under ARD conditions was the *ethylene-responsive transcription factor 1B-like (ERF1B)*. In a second experiment M26 plantlets were exposed to different abiotic stressors, namely heat, salt and nutrient starvation, and candidate gene expression was determined in the roots. The expression levels of *BIS3* and *B4Hb* were highly and specifically upregulated in ARD soil, but not upon the abiotic stress conditions, whereas *ERF1B* also showed higher expression under heat stress. In conclusion, *BIS3* and *B4Hb* are recommended as early ARD biomarkers due to their high expression levels and their high specificity.

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Introduction

Apple replant disease (ARD) presents a serious economic risk in orchards and tree nurseries where apple trees are frequently replanted [1, 2]. Characterized by its distinctive symptoms of reduced shoot growth, stunting, shortened internodes, root damage and root tip necrosis [3–5], ARD leads to reduced and delayed fruit yields as well as reduced fruit quality [1, 2]. The estimated yield losses caused by ARD range from 20–50% [6]. Eventually, ARD can render a site unprofitable for apple cultivation [7, 8]. Due to intensification of apple cultivation to certain areas accompanied by an increase of planting density, the problem of ARD has increased over the last decades. Crop rotation systems and soil exchange are usually difficult to employ in apple cultivation, mainly due to the high degree of technical specialization in apple growing sites and the perennial nature of the trees [9].

Numerous potential causal agents of ARD have been identified over the years. The growth-reducing effect of ARD can be abolished by disinfection of the affected soil, impressively demonstrating the biotic nature of the main causes of ARD [e.g. 10–12]. Among these an increase of detrimental oomycetes (*Pythium* [1, 2, 13] and *Phytophthora* [1, 13]) fungi (*Cylindrocarpon* [1, 2, 14–16] and *Rhizoctonia* [1, 13, 15]) and nematodes like *Pratylenchus penetrans* [10, 17–20] have been mostly attributed. Along with this, a decrease in beneficial soil organisms such as fluorescent *Pseudomonas* species has been reported [21, 22]. The abundance as well as the activity of these biotic factors can be influenced by abiotic factors such as soil texture, organic matter and pH [23, 24]. Overall, ARD presents a complex phenomenon which can best be characterized as a dysbiosis or negative plant-soil feedback. This is reflected in the most recent definition of Winkelmann et al. [9], which attributes the detrimental effect of the previous apple culture on the replant generation to a harmful shift in soil-borne (micro)organism communities.

The major counteractions employed against ARD are chemical soil disinfection (fumigation), biofumigation using *Brassicaceae* plant material, inter-row planting and soil substitution [9]. Because of its detrimental effects on the environment, chemical soil disinfection is not available any longer to several European countries due to its discontinued registration or its application is strictly regulated. Thus, an integrated solution to overcome ARD is of higher interest than ever before. Novel approaches aim at using *Brassicaceae* plant parts and seed meal for a biological relief from the ARD agents, anaerobic soil disinfestation or the incorporation of beneficial microorganisms to the soil to fight the disease [21, 25–30]. Apple genotypes with an increased tolerance towards ARD have been described [31, 32] and a long-term aim is breeding for ARD-resistant rootstocks. Until now, however, no feasible counteraction is available.

In order to develop sustainable countermeasures against ARD, its underlying complex causes have to be unraveled on the soil side but also inside the plant. Recently, Weiß et al. [11] examined the transcriptomic response in the roots of the ARD-sensitive apple rootstock M26 (hereafter indicated as M26) [10, 26] and observed a down-regulation of primary metabolism genes. At the same time, genes involved in secondary metabolite production, plant defense and regulatory and signaling genes were upregulated in response to ARD. Among the genes with a function in the secondary metabolism, those involved in phytoalexin biosynthesis were strongly upregulated. In addition, high amounts of the products of this biosynthesis pathway, namely 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzufuran, 2'-hydroxyaucuparin and noreriobofuran, could also be detected in the ARD-affected roots [33]. These compounds are part of the biotic stress response and have been described to act in particular against fungal pathogens [34], which have been attributed an enhanced role in ARD [2, 16].

Interest is given in biomarkers that allow an early detection and possibly also a quantification of the reaction of apple to ARD. These biomarkers would not only support breeding approaches for ARD tolerance but also allow to evaluate the efficacy of newly developed management options. In this study, seven genes that were identified to be strongly regulated in apple roots upon contact with ARD-affected soil [11], i.e. *1-aminocyclopropane-1-carboxylate oxidase homolog 1-like*, *ethylene-responsive transcription factor RAP2-11-like*, *ethylene-responsive transcription factor 1B-like*, *gibberellin-regulated protein 1-like*, *zinc finger domain-containing protein 10-like*, *biphenyl synthase 3* and *biphenyl 4-hydroxylase*, were chosen to be tested for their suitability as potential transcriptional biomarkers for an early detection of ARD. Gene expression was analyzed in apple roots growing on three different either untreated or γ-irradiated ARD-affected soils to provide a comparison to disease-free conditions, and was complemented by phytoalexin analysis. In a first experiment, the comparison of ARD-affected soils of different soil texture and chemical characteristics allowed distinguishing universal ARD responses from soil-specific responses regarding candidate gene expression. The promising biomarker candidates resulting from this approach were then tested in a second experiment for their specificity. Therefore, apple plants were exposed to different abiotic stressors, such as heat, salt stress, and nutrient starvation, and candidate gene expression was determined in the roots. Since the disinfected γ-irradiated ARD-affected soil can be regarded as a rather artificial control, the specificity test also included virgin (healthy) soils from plots adjacent to three ARD plots were included as an additional control.

Material and methods

Soil origin and disinfection

For the first experiment (candidate gene identification), soils from the three BonaRes-ORDIAmur ARD reference sites (www.ordiamur.de) in Germany were sampled in a depth of 0–20 cm in January 2017: Ruthe (Leibniz University Hannover, Sarstedt, 52˚14'39.8"N 9˚49'08.2"E), Heidgraben (Baumschule Harald Klei, Heidgraben, 53˚41'57.5"N 9˚40'59.6"E), and Ellerhoop (Gartenbauzentrum Schleswig-Holstein, Ellerhoop, 53˚42'51.9"N 9˚46'13.0"E). These soils were chosen to represent different soil textures. The Ruthe soil is of a loamy texture, while soil from Heidgraben is of a very sandy consistency and Ellerhoop soil is a loamy sand [12]. The soils were homogenized by sieving through an 8-mm mesh. One aliquot of each soil volume was packed in autoclavable plastic bags at a volume of 12 L each and sent for γ-irradiation with a minimum dose of 10 kGy (recorded dosages: 11.16 kGy minimum, 32.81 kGy maximum, Beta Gamma Service, Wiehl, Germany). The remaining untreated soil was transferred to buckets, covered with breathable MyPex fabric (Don & Low Limited, Angus, Scotland) and stored at outdoor temperature for approximately one month during which the γ-irradiation took place. The untreated soil will be referred to as ARD and the γ-disinfected soil as γARD. At the start of the experiment, all soil variants were supplemented with 2 g L^{-1} of the slow-release fertilizer Osmocote Exact Standard 3–4 M (16% total nitrogen, 9% phosphorus pentoxide, 12% potassium oxide, 2% magnesium oxide + trace elements, Everris International B.V., Geldermalsen, The Netherlands, https://icl-sf.com/global-en/products/ornamental_horticulture/8840-osmocote-exact-standard-3-4m).

In the second experiment (candidate gene validation), salt and heat stressed plants were potted into a mix of peat substrate and sand (Steckmedium, Klasmann-Deilmann GmbH, Geeste, Germany, with perlite (1–1.7 mm), white peat (0–7 mm) and white sod peat (1–7 mm) + sand, 2 + 1, 60 mg L^{-1} nitrogen, 70 mg L^{-1} phosphorus, 120 mg L^{-1} potassium, 85 mg L^{-1} magnesium, 60 mg L^{-1} sulfur). Peat substrate mixed with sand without any additional stress served as a control. All substrate variants (heat, salt stress and peat substrate) were fertilized

Table 1. Overview of the treatments utilized for experiment 1 and 2 (candidate gene validation). Fertilized variants were supplemented with 2 g L^{-1} Osmocote Exact 3–4 M before filling of the pots. Peat substrate refers to Steckmedium by Klasmann-Deilmann GmbH, Geeste, Germany (see above).

	Abbreviation	Substrate / soil	Treatment / specifications
Experiment 1	ARD Ellerhoop	ARD soil Ellerhoop	Fertilized
	γARD Ellerhoop	ARD soil Ellerhoop	γ-irradiated, fertilized
	ARD Heidgraben	ARD soil Heidgraben	Fertilized
	γARD Heidgraben	ARD soil Heidgraben	γ-irradiated, fertilized
	ARD Ruthe	ARD soil Ruthe	Fertilized
	γARD Ruthe	ARD soil Ruthe	γ-irradiated, fertilized
Experiment 2	ARD	ARD soil Heidgraben	Fertilized
	Grass	Control soil Heidgraben	Fertilized
	Peat (substrate)	Peat substrate + quartz sand (2 + 1)	Fertilized
	Heat	Peat substrate + quartz sand (2 + 1)	fertilized, plants 3 days at 37˚C
	Salt	Peat substrate + quartz sand (2 + 1)	one-time application of 50 mL 0.17 M NaCl (10 g L^{-1}) at the start of the experiment, fertilized
	Nutrient starvation	Quartz sand	not fertilized

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with 2 g L^{-1} Osmocote Exact Standard 3–4 M as described above. Nutrient starvation was applied by potting the plants into unfertilized quartz sand. Further variants included soils from ARD and grassland plots from Heidgraben as references representing field conditions. In the grassland plots within the reference sites, no members of the Rosaceae had been grown. Thus, this soil served as a control soil that was not affected by replant disease but contained microorganism communities native to the site. The soils were sieved and fertilized as described above. All variants of experiments 1 and 2 with their respective substrates and stress treatments are depicted in Table 1.

The sampling and evaluation schedules of for both experiments are presented in S1 Table.

Plant cultivation and sampling

For both experiments, plants of the ARD sensitive apple rootstock M26 were clonally propagated *in vitro* via axillary shoots on a modified MS medium [35] containing 3% sucrose, 0.5 μM indole-3-butyric acid (IBA) and 4.4 μM 6-benzylaminopurine (BAP). They were grown at 24˚C and 16 h light / 8 h darkness provided by Philips MASTER TL-D 58W/865 fluorescence tubes at a photosynthetically active photon flux density (PAR) of 25–30 μmol m^{-2} s^{-1}. *In vitro* rooting was induced on ½ MS medium supplemented with 2% sucrose and 4.92 μM (IBA) [11]. The rooted plants were transferred to substrate for cutting propagation (Steckmedium, Klasmann-Deilmann GmbH, see above) and kept under a foil tent in the greenhouse for acclimatization. After two weeks, subsequently increasing ventilation of the tent was started until the plants were fully adapted to greenhouse conditions.

After approximately four weeks in the greenhouse the plants were transferred into the test soils (see Table 1). The first experiment used the ARD soils from the three sites Ruthe, Heidgraben and Ellerhoop (see above) either untreated or disinfected by γ-irradiation. The second experiment aiming at specificity testing used either ARD soil from Heidgraben, peat substrate (Steckmedium, Klasmann-Deilmann GmbH + sand, 2 + 1) or sand alone (Table 1). The different variants were prepared as follows: For salt stress, the plants were potted into fertilized peat substrate (Steckmedium, Klasmann-Deilmann GmbH + sand, 2 + 1, + 2 g L^{-1} Osmocote) and initial watering was carried out with 50 mL of a 0.17 M NaCl solution per pot. From then on, irrigation was carried out with regular tap water. For the heat treatment, the plants were potted into fertilized peat substrate and grown for four days in the greenhouse. Thereafter, they were transferred to a culture cabinet (Rubarth Apparate GmbH, Laatzen, Germany) for three days

at 37˚C and a 16 h photoperiod, after which they were placed back into the greenhouse. Nutrient starvation was induced to the plants with unfertilized quartz sand. Controls included soil from the Heidgraben reference site from the ARD patches (ARD, positive control) and from patches covered in grass (Grass soil, negative control) as well as fertilized peat substrate-sand mix (substrate control, second negative control).

Round pots of 0.46 L volume and 10.5 cm diameter were used for the plants sampled after 1, 3, and 7 days in the first experiment and all plants in the second experiment. Additionally, 1 L pots were used for plants sampled after 8 weeks in the first experiment. All pots were lined with MyPex fabric to avoid washing out of soil or substrate during irrigation. They were placed in the greenhouse in a randomized design. Cultivation during the first three days was carried out without additional lighting. From then on, additional light was provided by SON-T Philips Master Agro 400 W lamps (Hamburg, Germany) if solar radiation fell below 25 klx to provide 16 h of daylight and thus comparable growing conditions over the whole year. The temperature in the greenhouse chamber was 21.1 ± 1.3˚C and the relative air humidity 58.1 ± 7.9% during the first experiment and 22.6 ± 3.1˚C and 64.2 ± 10.6%, respectively, for the second experiment. Plants were irrigated by hand on a daily basis and plant protection was carried out according to horticultural practice.

For the first experiment (candidate gene identification), complete root systems were harvested for gene expression analysis at day 0 (acclimatized plants before potting) and 1, 3 and 7 days after potting into the experimental soils. For this, 20 plants per soil variant and sampling day were selected randomly. They were unified into four pooled samples consisting of five plants each (S2 Table). For the second experiment (candidate gene validation), sampling was carried out after 7 and 14 days. Complete root systems of 15 plants per variant and time point were harvested for gene expression analysis yielding five pooled samples consisting of three individual plants each (S3 Table). After the seven-day sampling point in the second experiment, the heat-stressed plants were moved to the greenhouse until the end of the experiment. Therefore, these plants were no longer exposed to the heat at the second sampling point.

The plants were quickly but gently washed with tap water, blotted dry briefly and the complete root system was separated from the shoot, transferred to 2 mL reagent tubes (Sarstedt, Nümbrecht, Germany) and frozen in liquid nitrogen. Storage took place at -80˚C until RNA extraction. In both experiments, the shoot length was measured weekly. At the final day of sampling, the roots were separated from the shoots and the fresh masses of shoots and roots were recorded. Shoots and roots were then frozen at -20˚C and freeze dried for three days (Christ ALPHA 1–4 LSC, Osterode, Germany) to determine the dry mass. The freeze-dried samples were stored above silica gel (Carl Roth, Karlsruhe, Germany) until preparing for phytoalexin analysis (see below). In the second experiment, dry mass was recorded after oven-drying for three days at 80˚C.

RNA extraction and first strand cDNA synthesis

Within the variants, pooling for both experiments (experiment 1: n = 4 pooled samples = biological replicates, see S2 Table, experiment 2: n = 5 pooled samples = biological replicates, see S3 Table) took into account that the mean shoot length of the pooled plants was comparable. The pooled root systems were homogenized at 29 Hz for 1 min using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) cooled with liquid nitrogen. RNA extraction from 100 mg fresh mass of ground root powder was carried out with the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) according to the manufacturer's instructions. The included extraction buffer for phenol-containing plants was used (RP lysis buffer) and 40 μL of the provided elution buffer. Genomic DNA was removed from the RNA via digestion

with DNase I (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. The concentration and quality of the obtained RNA was determined spectrophotometrically (NanoDrop 2000c, Peqlab, Erlangen, Germany) and the quality was checked on a 1% agarose gel. The isolated RNA was stored at -80˚C until first strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with an input of 1 μg RNA and with random hexamer primers. The resulting cDNA was aliquoted for the qPCR measurements and stored at -20˚C until then.

Quantitative PCR

All reactions were carried out on a CFX Connect™ cycler (Bio-Rad, Hercules, CA, USA) using the SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primers used (Table 2) had been previously validated for the apple rootstock genotype M26 by Weiß et al. [11]. As reference genes, elongation factor 1-α (*EF1a*), elongation factor 1-β (*EF1b*) and tubulin beta chain (*TUBB*) [11] were selected after testing their expression stability between ARD and γARD variants (Table 2) at each time point for experiment 1 and between all six variants in experiment 2. Each forward and reverse primer were used in a concentration of 20 nM and amplification efficiency was determined for each experiment separately. A pool of all cDNA samples in equal amounts was created and the quantification cycles (c_qs) for the dilutions 1:5, 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:2000 were determined. The protocol used for the efficiency tests and the following qPCRs was as follows: 3 min at 95˚C, followed by 10 s at 95˚C and 30 s at 60˚C for 40 cycles and a melt curve analysis (65˚C to 95˚C for 5 s each with an increment of 5˚C). The amplification efficiencies for each primer pair were calculated with the CFX manager

Table 2. Primer sequences and amplicon lengths of candidate and reference genes used in RT-qPCR analyses [11]. MDP ID: Malus domestica predicted gene ID [38]. Amplification efficiency in % (E [%]) with corresponding coefficient of correlation (R^2). n.a. = not analyzed, excluded from the second experiment.

Gene name (MDP ID)	Abbreviation	Primer sequence 5'– 3'	Amplicon length [bp]	experiment 1		experiment 2	
				E [%]	R^2	E [%]	R^2
1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (MDP0000314499)	*ACO1*	f: CGCAGTTGGAGATGAACTTG	167	98.2	0.995	n.a.[1]	n.a.
		r: CATGCCGTGATGGACAGTAG					
ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)	*ERF RAP2.11*	f: TTCCAACAGCCGAAGCAAG	169	71.3	0.980	n.a.	n.a.
		r: CTTTGATCTCAGCAACCCATCTC					
ethylene-responsive transcription factor 1B-like (MDP0000127134)	*ERF1B*	f: GTCACCTGAATCTTCGTTTG	121	90.8	0.994	96.5	0.986
		r: GGAAATCAGACCGTAGAGAAG					
gibberellin-regulated protein 1-like (MDP0000140078)	*GASA1*	f: CGTTGCAGCTGTGTTCCTC	156	92.5	0.993	n.a.	n.a.
		r: CATCTGCATGCCCGAATATGAG					
zinc finger domain-containing protein 10-like (MDP0000922823)	*GATAD10*	f: GCTCGTTTCTGGAGGAGTC	153	91.3	0.983	n.a.	n.a.
		r: GATTCCCGCTGTCGTAGAATC					
biphenyl synthase 3 (MDP0000287919)	*BIS3*	f: GGCAAGAAGCAGCATTGAAAG	105	97.8	0.997	94.6	0.998
		r: CACAACCTGGCATGTCAAC					
biphenyl 4-hydroxylase (MDP0000152900)	*B4Hb*	f: GCTGAGTATGGCCCGTATTG	156	98.7	0.996	96.7	0.999
		r: AGGAACCCGTCGATTATTGG					
elongation factor 1-alpha (MDP0000304140)	*EF1a*	f: GAACGGAGATGCTGGTATGG	159	94.5	0.997	94.9	0.998
		r: CCAGTTGGCTCCTTCTTCTC					
elongation factor 1-beta 2-like (MDP0000903484)	*EF1b*	f: GAGAGTGGGAAATCCTCTG	138	100.1	0.994	95.4	0.998
		r: ACCAACAGCAACCAATTTC					
tubulin beta chain (MDP0000951799)	*TUBB*	f: TTCTCTGGGAGGAGGTACTG	147	99.8	0.998	90.6	0.998
		r: GTCGCATTGTAAGGCTCAAC					

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software version 3.0 according to Pfaffl [36] for both experiments separately. Only primers with an efficiency between 90 and 110% [37] were used for the following analyses leading to an exclusion of ethylene-responsive transcription factor RAP2-11-like (*ERF RAP2.11*) (Table 2). Gene expression analysis was performed in two technical and four (experiment 1) or five (experiment 2) biological replicates per time point and soil variant. Normalized gene expression was calculated according to Pfaffl [36].

Phytoalexin extraction and analysis

Complete root systems were harvested at different time points to measure total phytoalexin contents and correlate them to expression of genes involved in phytoalexin biosynthesis and growth parameters (S1 Table). The samples for phytoalexin analysis were taken as follows for experiment 1: At days 0, 3, 7, 10 and after 8 weeks (day 56) in replicates of 10 single plants per treatment, which were later unified to obtain a minimal dry mass of 42.2 mg for the analyses (S2 and S4 Tables). For the second experiment, subsets of the 14 days gene expression samples (n = 5 pooled samples) were analyzed (n = 5 individual plants, S3 Table). The dried roots were ground to a fine powder using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) at 29 Hz for 1 min.

Extraction of total phytoalexins was carried out as described previously [39]. In short, 1 mL of methanol supplemented with 50 μg of 4-hydroxybiphenyl (internal standard for relative quantification) were added to each samples in a 2 mL reagent tube. The samples were vortexed continuously with Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) at the maximum speed of 2.700 rpm. The resulting extracts were centrifuged at 13,000 rpm for 10 min and the supernatant was transferred into a new tube. A 200 μL aliquot of supernatant was transferred to a new 1.5 mL reagent tube. After drying under an air stream, the residue was re-dissolved in 200 μL ethyl acetate and centrifuged at 13,000 rpm for 10 min. The clear supernatant was transferred to a GC-MS vial with glass inlet. The ethyl acetate was evaporated by air stream and the residue was silylated with 50 μL N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Alfa Aesar, Thermo Fischer, Kandel, Germany) at 60˚C for 30 min. Silylated samples were analyzed by gas chromatography—mass spectrometry (GC-MS) using the following temperature program: 70˚C for 3 min, then linear increase of temperature from 70˚C to 310˚C over 24 min (10˚C min^{-1}) and finally 310˚C for 5 min. Helium was the carrier gas with a flow rate of 1 mL min^{-1}. The injection volume was 1 μL with split ratio 1:10. One technical replicate was measured per sample (biological replicate) after testing the stability of the system with two injections per sample. Furthermore, technical reproducibility was ensured by repeated measurements of the alkane standards at the beginning and the end of the sequences and additionally in the middle if the sequence contained more than 40 samples. At the same time, the alkanes served to calculate the retention index (RI). Quantification of individual compounds was done based on the internal standard 4-phenylphenol. A response factor of 1 was assumed for all compounds.

Nutrient analysis

Nutrients were analyzed in the oven-dried leaves of plants from the second experiment sampled after 8 weeks. Leaves from the top, middle and bottom region of each shoot were collected. Material from one plant each was collected, which yielded 5 biological replicates, except for salt stress, where only four plants were available (S3 Table). Nutrients in each of these samples were measured in one technical replication. The samples were ground to a fine powder using 50 mL grinding vessels of a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) at 30 Hz for 1 min. Fifty mg of each homogenized sample were transferred into 50 mL glass

vessels. They were incinerated over night at 480˚C until the ash was greyish-white. After cooling down of the ashes, 1 mL of extraction solution (1.5% w/v hydroxylammonium chloride in 6 M HCl) was added to each sample. After 10 minutes of incubation, 9 mL of deionized water were added. The solution was thoroughly shaken and filtered through a blue band filter. The following elements were analyzed: zinc, boron, barium, strontium, aluminum, iron, manganese, calcium, magnesium, phosphorus, sodium and potassium. The measurements took place on an inductively coupled plasma optical emission spectrometer (ICP-OES; Varian 725-ES, Agilent Technologies, Santa Clara, CA, USA).

Total carbon and nitrogen contents were analyzed in 5–7 mg of the oven-dried and homogenized leaf material after dry combustion on a Vario EL III elemental analyzer (Elementar Analysensysteme, Hanau, Germany). The high organic standard (HOS; IVA Analysentechnik, Meerbusch, Germany) with 7.45% C and 0.52% N was used as a reference (charge 287371/264236).

Statistical analyses

All statistical analyses were conducted with the statistics software R version 3.5.1 [40] in RStudio version 1.1.456. For experiment 1, the influence of soil texture (soil origin) and soil treatment on shoot length after eight weeks, shoot and root dry mass and their interaction was investigated by a two-way analysis of variance (ANOVA). Differences of normalized gene expression between untreated ARD soil and γ-irradiated ARD soil of the first experiment were analyzed using Student's t-Test for each soil and time point separately. For the second experiment, a linear model was fitted for the expression data of each gene of interest, considering each time point separately. Multiple comparisons were calculated using a Tukey test within the "multcomp" package version 1.4–10 [41]. Shoot lengths were analyzed separately by week. A linear model was fitted and differences between the soil treatments were assessed with a Tukey-Duckworth test for $p < 0.05$ using the "multcomp" package.

Phytoalexin data were evaluated with all-pairs interaction contrast analysis using sandwich estimator (Tukey test) in R with the packages "sandwich" [42] and "multcomp" [41] and $p < 0.05$ for the compact letter display. For the data containing multiple zeros (non-detected amounts of phytoalexins), nonparametric multiple comparisons for relative contrast effects were applied within the R package "nparcomp" version 3.0 [43].

Significant differences for the nutrient analysis were evaluated using linear models and least square means in R. The correlation analysis of the relationship between two parameters was performed using linear regression analysis. The Pearson correlation coefficient, r, was calculated using Sigma Plot 11.0 (Systat Software Inc., San Jose, USA). Statistical significance (P-value) was tested using one-way univariate ANOVA with $p < 0.05$.

Results

The following section separately describes the results of the two experiments, starting with data of candidate gene identification (experiment 1) and followed by results from candidate gene validation (experiment 2).

Experiment 1: ARD affected soils influenced growth of apple rootstock M26 to different extents

The three ARD soils tested in this experiment influenced plant growth to different extents, with the sandy soil Heidgraben having the strongest overall impact. After eight weeks, the strongest reduction in shoot growth was visible in soil from Heidgraben (34.9 ± 4.1 cm in γARD to 10.5 ± 5.7 cm in ARD), followed by Ruthe (37.6 ± 4.7 cm in γARD to 27.4 ± 7.4 cm in

ARD) and Ellerhoop (22.7 ± 7.0 cm to 16.7 ± 5.8 cm). In soil from Ellerhoop, shoot length did not differ significantly from week 7 on and individual plants even had a greater shoot length in ARD soil compared to γARD (Fig 1). The ANOVA showed a significant influence of both the soil origin and soil treatment (untreated, disinfected) on shoot length after 8 weeks ($p < 0.001$ for both). Additionally, shoot length was affected differently by the combination of soil texture and treatment, significantly as seen by a p-value of 1.884e-05 for the interaction of the two parameters.

All ARD variants negatively affected the root system, showing a darker coloration and considerably less branching and biomass (Figs 1 and 2). Browning and blackening of the roots was also visible for the plants showing only little to no reduction in shoot growth. In general, plants grown in Ruthe soil achieved the longest shoots, followed by Heidgraben and Ellerhoop.

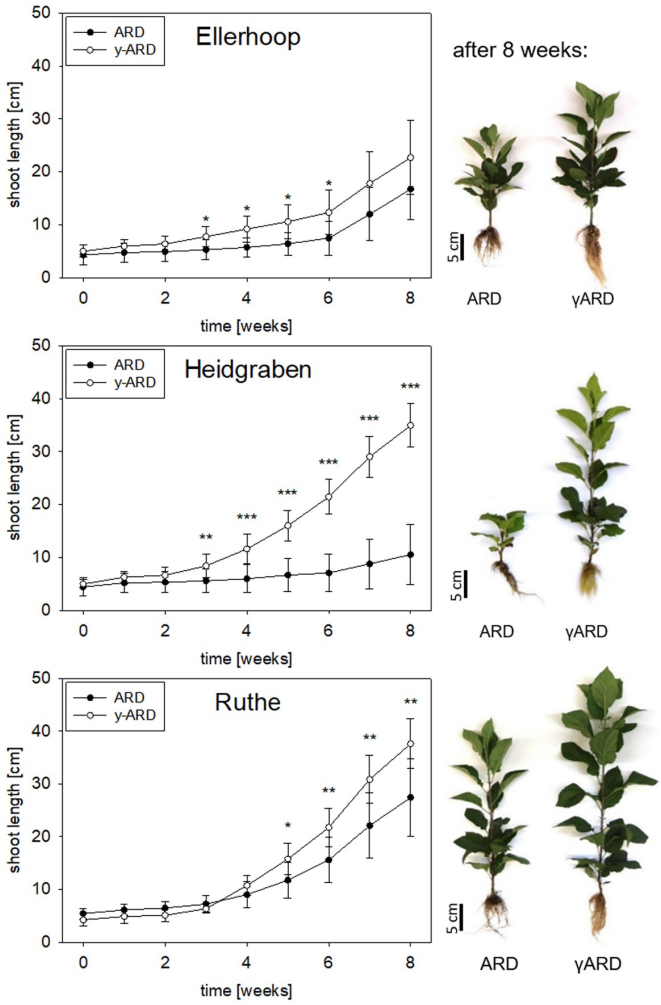

Fig 1. Growth of M26 plants in untreated ARD soil (ARD) and γ-irradiated ARD soil (γARD) from the three sites Ellerhoop, Heidgraben and Ruthe over eight weeks. Photos depict representative plants of each variant at the end of the experiment. Means ± SD, n = 10, Tukey-Duckworth test, significant differences at each time point shown for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

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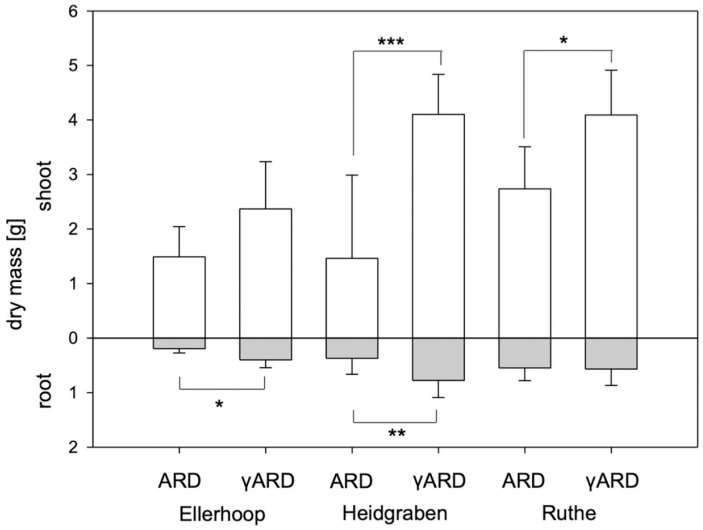

Fig 2. Shoot and root dry masses of M26 plants after 8 weeks of cultivation in ARD or γARD soil from the three sites Ellerhoop, Heidgraben and Ruthe. Means ± SD, n = 10, differences between ARD and γARD assessed with Tukey-Duckworth test for each soil ($p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

https://doi.org/10.1371/journal.pone.0238876.g002

The pattern of shoot dry mass followed that of the shoot length (Fig 2). The strongest reduction in shoot dry mass of ARD *versus* γARD plants was present in Heidgraben, followed by Ruthe and Ellerhoop, where the reduction was not statistically significant after 8 weeks. The root dry masses on the other hand showed a significant reduction in Heidgraben and Ellerhoop soil, but no reduction was observed for plants grown in soil from Ruthe (Fig 2). Both shoot and root dry mass were significantly influenced by both soil and treatment ($p < 0.001$ for both shoot dry mass and $p < 0.001$ and $p < 0.05$ for root dry mass respectively). An interaction between soil texture and disinfection treatment was again present for shoot dry mass with $p = 0.012$.

Experiment 1: The transcription factor *ERF1B* and the phytoalexin biosynthesis genes *BIS3* and *B4Hb* showed distinct early differences between γARD and ARD variants

The four potential biomarker genes for early detection of ARD, *1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (ACO1)*, *gibberellin-regulated protein 1-like (GASA1)* and *GATA zinc finger domain-containing protein 10-like* (*GATAD10*) were not distinctly expressed between the γARD and ARD variants in the three ARD soils investigated (S1 Fig). *ACO1* showed a slight trend of higher expression in the γARD variants, which was significant at day 3 in the soil from Ruthe and at day 7 in Ellerhoop soil. In general, the *ACO1* transcript was of low abundance in all variants (S1 Fig). The *GATAD10* transcript of the ARD variant showed a peak on day 1. This was due to only one of the four biological replicates each from Ruthe and Heidgraben showing a considerably higher *GATAD10* expression than the other three replicates, as indicated by the high standard deviation.

The *ethylene-responsive transcription factor 1B-like* (*ERF1B*) showed significant differences in gene expression between the untreated and irradiated variants of all three ARD soils from day 3 on (Fig 3A). In Ellerhoop soil, a slight trend of higher upregulation was observed at day

7 in comparison to Ruthe and Heidgraben, where the expression was rather comparable between day 3 and 7.

A strong and fast gene expression response was found for the two phytoalexin biosynthesis genes *biphenyl synthase 3* (*BIS3*) and *biphenyl 4-hydroxylase* (*B4Hb*). A significant difference in gene expression between ARD and γARD was found for all soils after 3 days with a higher expression of both genes in the ARD variants. *B4Hb* was the only exception in Ellerhoop where the expression differed significantly only after 7 days (Fig 3B and 3C). The fastest response was observed in soil from Ruthe, where the BIS3 transcript reached a plateau already after 3 days. The highest *BIS3* expression was found after 56 days in soil from Ellerhoop. *B4Hb* expression showed similar patterns as *BIS3*, but on a lower overall expression level.

Due to their consistent upregulation in roots affected by ARD, the three genes *ERF1B*, *BIS3 and B4Hb* were further tested in experiment 2 for their specificity for ARD.

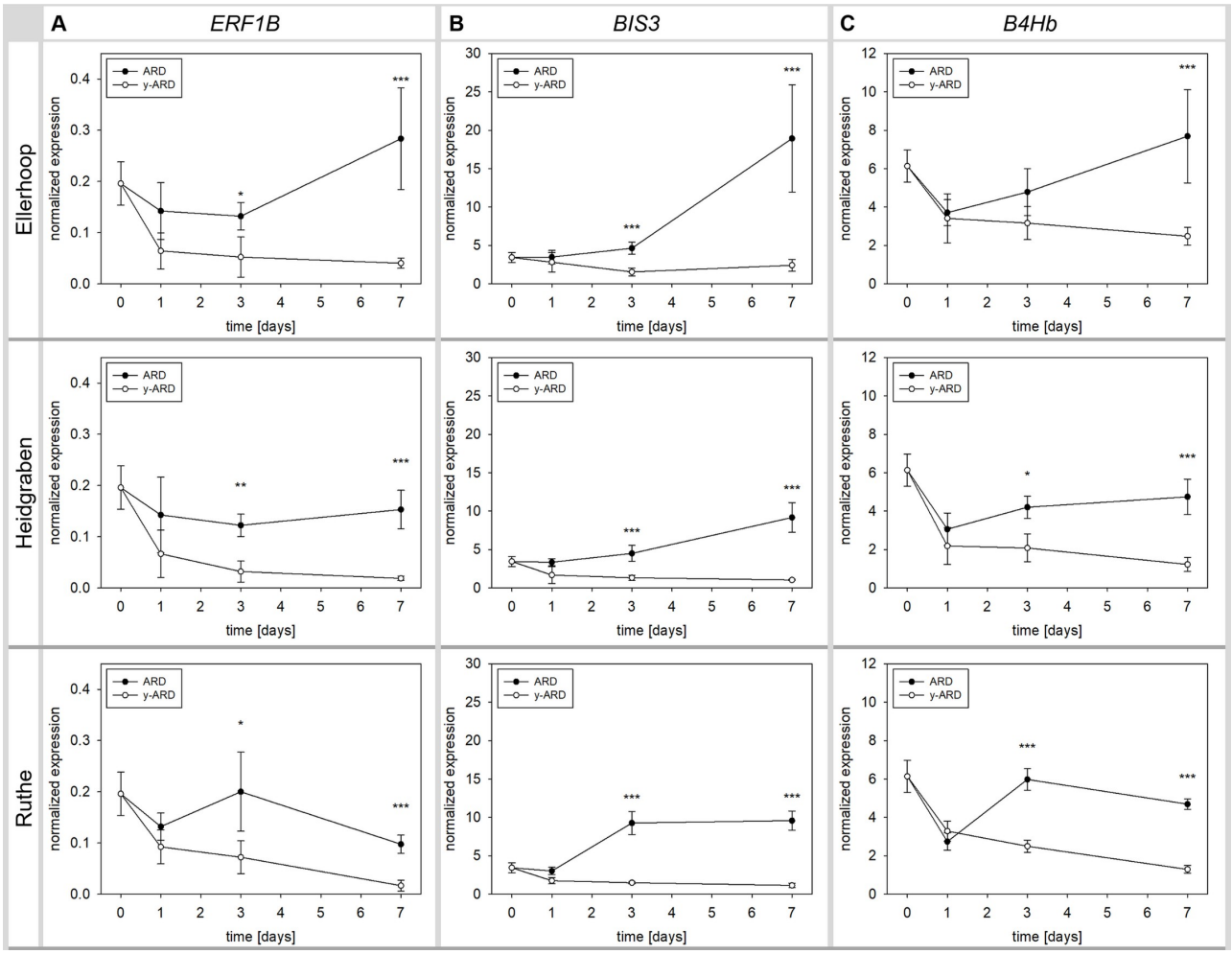

Fig 3. Normalized gene expression of *ethylene-responsive transcription factor 1B-like* (*ERF1B*), *biphenyl synthase 3* (*BIS3*) and *biphenyl 4-hydroxylase* (*B4Hb*) in M26 roots growing in ARD and γARD soil from the three sites Ellerhoop, Heidgraben and Ruthe. Means ± SD, n = 4 pooled samples. Significant differences at each time point shown for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) as investigated by Tukey tests.

https://doi.org/10.1371/journal.pone.0238876.g003

Experiment 1: Phytoalexin contents were elevated in the root shortly after potting into untreated ARD soil

Total phytoalexin contents in roots grown in ARD soils for 56 days were significantly increased compared to those grown in γARD soils from all three locations (Fig 4). The highest total phytoalexin content was found in roots grown in soil from Heidgraben with an average content of 1.082 mg g^{-1} DW. In all three soils, the total phytoalexin contents increased rapidly during the initial days they were still high at day 56 (S3 Fig and Fig 4). The total phytoalexin content in the roots correlated strongly with shoot dry mass ($p < 0.0001$) and less pronounced with root dry mass ($p < 0.05$).

Among the four biphenyls and four dibenzofurans identified by GC-MS, 2-hydroxy-4-methoxydibenzofuran (RI = 2131) was the only phytoalexin that was detected in all samples including day 0. The average content of 2-hydroxy-4-methoxybiphenyl in roots grown for 56 days on γARD soil was 0.014 mg g^{-1} DW, whereas it reached to 0.657 mg g^{-1} DW in roots grown in ARD soils for 56 days, which corresponds to a 47-fold increase. Also, all other seven phytoalexin compounds were induced in the ARD variants (Fig 4). Noraucuparin (RI = 2121) and noreriobofuran (RI = 2259) were the most strongly induced biphenyl and dibenzofuran compounds, respectively, in roots from ARD soils, with a significant increase in Ellerhoop and Ruthe ARD variants (Fig 4). These individual compounds showed a significant negative correlation with shoot dry mass: noraucuparin ($p = 0.0001$), noreriobofuran ($p < 0.001$), hydroxyeriobofuran ($p < 0.001$), eribofuran ($p < 0.05$) and 2-hydroxy-4-methoxydibenzofuran ($p = 0.0001$). Furthermore, noraucuparin ($p < 0.05$), 2-hydroxy-4-methoxydibenzofuran ($p < 0.01$), and eribofuran ($p < 0.05$) were negatively correlated with the root dry mass.

Experiment 2: Abiotic stressors influenced plant growth and nutrient composition

For experiment 2, plants were grown under different abiotic stress conditions (heat, salt and nutrient starvation) and in a "healthy" control soil from a grass plot (grass) (Table 1) to further characterize the selected early ARD biomarker candidate genes *ERF1B*, *BIS3 and B4Hb* from experiment 1. Plants showed characteristic symptoms related to the applied stresses. The lack

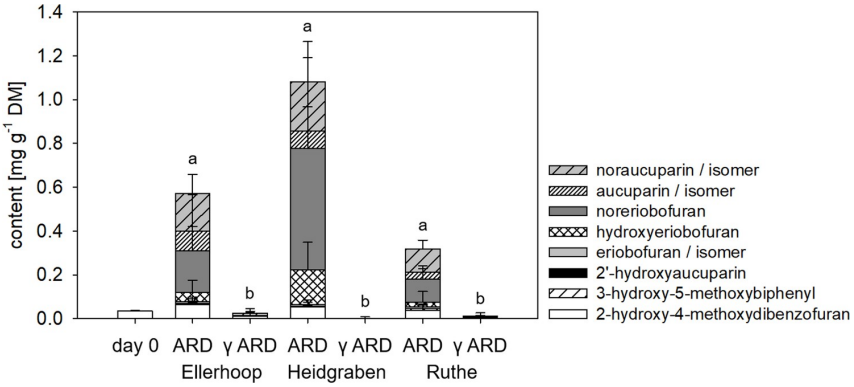

Fig 4. Means and standard deviations of individual phytoalexin compounds measured in M26 roots grown in ARD or γARD soil from the sites Ellerhoop, Heidgraben and Ruthe after 8 weeks. n = 7 for Ellerhoop ARD, n = 8 for Ellerhoop γARD, n = 6 for Heidgraben and Ruthe. Different letters indicate significant differences in total phytoalexin content as calculated by multiple comparisons (Tukey test, $p < 0.05$). The results of the statistical comparisons of the individual compounds are given in Table 3 below.

https://doi.org/10.1371/journal.pone.0238876.g004

Table 3. Results of all-pairs interaction contrast analysis using sandwich estimator of individual phytoalexin compounds measured in M26 roots grown in ARD or γARD soil from the sites Ellerhoop, Heidgraben and Ruthe after 8 weeks (see Fig 4). Different letters indicate significant differences in content as calculated by multiple comparisons (all-pairs interaction contrast analysis using sandwich estimator or nonparametric multiple comparisons for relative contrast effects, $p < 0.05$).

Phytoalexin compound	Ellerhoop		Heidgraben		Ruthe	
	ARD	γARD	ARD	γARD	ARD	γARD
2-hydroxy-4-methoxydibenzofuran	a	bc	ab	c	ac	c
3-hydroxy-5-methoxybiphenyl	a	a	a	a	a	a
2'-hydroxyaucuparin	a	a	a	a	a	a
eriobofuran	a	a	a	a	a	a
hydroxeriobofuran	ab	ab	a	b	ab	b
noreriobofuran	a	b	ab	b	a	b
aucuparin	a	a	a	a	a	a
noraucuparin	a	b	ab	b	a	b

https://doi.org/10.1371/journal.pone.0238876.t003

of nutrients especially inhibited both shoot and root biomass in the nutrient starvation variant and led to stunting and leaf chlorosis (Fig 5 and S4 Fig). The oldest leaves of the heat stress variant showed light brown angular necrotic spots as heat stress symptoms (S4 Fig). When the plants were transferred to the greenhouse, newly grown leaves did not show these specific symptoms anymore. The plants subjected to salt stress showed symptoms of leaf margin necrosis at the oldest leaves (S4 Fig). As watering was continued with regular tap water, the newly grown leaves did not show such symptoms anymore. Plants grown in ARD soil showed shortened internodes and darkened roots. In comparison to the variants grown in peat substrate, both variants grown in soil, ARD and grass, showed a browning of roots and reduced growth (Fig 5 and S4 and S5 Figs). Growth inhibition by the abiotic stresses was also reflected in a significant reduction in both shoot and root dry mass after 8 weeks (Fig 5).

A nutrient analysis of the plants from experiment 2 was conducted to test if (i) the nutrient starvation variant indeed caused deficient levels of nutrients in the plants and (ii) a possible connection between candidate gene expression and general nutrient status exists. All variants except the nutrient starvation variant were fertilized with Osmocote Exact 3–4 M (Table 1). Consequently, the nutrient starvation variant had significantly smaller contents of nitrogen (7.71 mg g^{-1} DM) and phosphorous (0.81 mg g^{-1}), which were approximately half the content of the other variants (Table 4). For potassium, however, contents were smaller in all non-soil

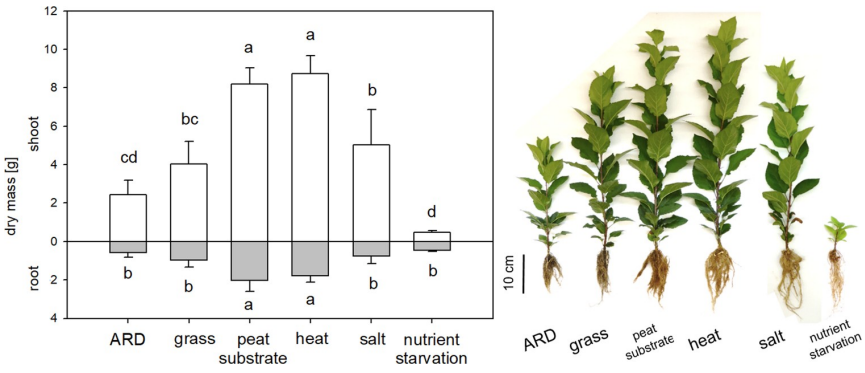

Fig 5. Shoot and root dry masses of M26 plants grown in the variants ARD soil, grass soil, peat substrate, heat, salt stress and nutrient starvation after 8 weeks (n = 5, except for salt stress n = 4). Different letters indicate a statistical difference (Tukey Test, $p < 0.05$) between variants. See Table 1 for details on the variants.

https://doi.org/10.1371/journal.pone.0238876.g005

Table 4. Nutrient contents [mg g^{-1}dry mass] in leaf material of M26 plants grown under the conditions ARD, grass soil, peat substrate, heat, salt stress and nutrient starvation as depicted in Table 1. Different lower case letters indicate significant differences between treatments (tested by estimated marginal means, p = 0.05), (means ± SD of n = 5, except for salt stress n = 4).

Content [mg g^{-1} DM]	ARD	Grass	Peat substrate	Heat	Salt	Nutrient starvation
Al	0.22 ± 0.05a	0.3 ± 0.06a	0.26 ± 0.04a	0.24 ± 0.03a	0.25 ± 0.04a	0.31 ± 0.08a
B	0.2 ± 0.06ab	0.27 ± 0.05a	0.25 ± 0.05a	0.21 ± 0.03ab	0.21 ± 0.02ab	0.15 ± 0.01b
Ba	0.16 ± 0.04ab	0.21 ± 0.05a	0.17 ± 0.03ab	0.16 ± 0.02ab	0.16 ± 0.02ab	0.12 ± 0.01b
C	480.63 ± 3.15a	478.97 ± 2.72a	477.35 ± 2.8a	478.37 ± 3.71a	477.41 ± 3.53a	459.33 ± 4.2a
Ca	7.58 ± 0.64a	7.18 ± 0.92a	8.87 ± 0.84a	8.1 ± 0.22a	8.43 ± 0.93a	13.39 ± 1.38b
Fe	0.06 ± 0.01a	0.09 ± 0.01ab	0.06 ± 0.01a	0.06 ± 0.01a	0.06 ± 0.02a	0.13 ± 0.05b
K	11.93 ± 1.82ab	12.88 ± 1.71a	7.36 ± 0.42c	7.5 ± 2.1c	9.47 ± 1.35bc	6.38 ± 0.71c
Mg	2.09 ± 0.18a	2.17 ± 0.14a	2 ± 0.25a	1.94 ± 0.2a	2.03 ± 0.12a	1.5 ± 0.19b
Mn	0.05 ± 0a	0.04 ± 0b	0.05 ± 0.01a	0.04 ± 0.01ab	0.04 ± 0b	0.04 ± 0b
N	18.14 ± 3.04a	17.27 ± 2.07ab	12.73 ± 1.16b	13.39 ± 2.55b	14.09 ± 2.7b	7.71 ± 1.69b
Na	2.23 ± 0.38ab	3.14 ± 0.38c	2.87 ± 0.46ac	2.61 ± 0.4ac	2.73 ± 0.59ac	1.59 ± 0.2b
P	1.62 ± 0.16a	1.88 ± 0.4a	1.69 ± 0.34a	1.66 ± 0.12a	1.8 ± 0.14a	0.81 ± 0.13b
Sr	0.016 ± 0.002ab	0.019 ± 0.003a	0.015 ± 0.001ab	0.014 ± 0.002b	0.014 ± 0.002b	0.037 ± 0.003c
Zn	0.04 ± 0a	0.04 ± 0a	0.03 ± 0bc	0.03 ± 0.01bc	0.02 ± 0c	0.04 ± 0.01ab

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based variants with 6.38 mg g^{-1} DM in nutrient starvation, 7.5 mg g^{-1} DM in heat stress, 9.47 mg g^{-1} DM in salt, and 7.36 mg g^{-1} DM in the peat substrate variant. Interestingly, the calcium content in the nutrient starvation variant was almost twice as high as in the other variants (Table 4).

Experiment 2: Expression of *BIS3* and *B4Hb* increased in ARD affected roots from week 1 to week 2, but was barely influenced by abiotic stressors

Gene expression of the two early ARD biomarker candidate genes belonging to the phytoalexin biosynthesis pathway, *biphenyl synthase 3* (*BIS3*) and *biphenyl 4-hydroxylase b* (*B4Hb*) showed a significantly higher normalized expression level in ARD soil compared to the abiotic stress conditions investigated (Fig 6A and 6B). Interestingly, a considerable expression of both *BIS3* and *B4Hb* was found in roots of the grass variant. At week 1, normalized expression between the grass and ARD variant was very similar. At week 2, *BIS3* and *B4Hb* expression remained at a similar level in the grass variant but was elevated in the ARD variant. For *BIS3*, the expression level in the heat, salt and nutrient starvation variants was significantly lower than in the ARD and grass variants for both time points. Gene expression in the abiotic stress variants did not differ significantly from the expression of the acclimatized plants (day 0) and the peat substrate variant (Fig 6A). *B4Hb* expression on the other hand was significantly higher in the heat and salt variants compared to the peat substrate variant at week 1, reaching a comparable level to the grass variant but being lower than in roots of the ARD variant (Fig 6B). At week 2, the *B4Hb* expression pattern resembled that of *BIS3*. Overall, these two biosynthetically linked genes followed almost the same expression pattern across all variants and time points, with *BIS3* being the more highly expressed gene.

The expression pattern of the *ethylene-responsive transcription factor 1B* (*ERF1B*) was prone to much higher variation in comparison to the highly expressed phytoalexin biosynthesis genes (Fig 6C). The high variation is likely caused by its low overall expression level. *ERF1B* expression followed a similar pattern as the two other candidate genes with a significant higher expression in the ARD variant at week 2 compared to the other variants. However, for *ERF1B*

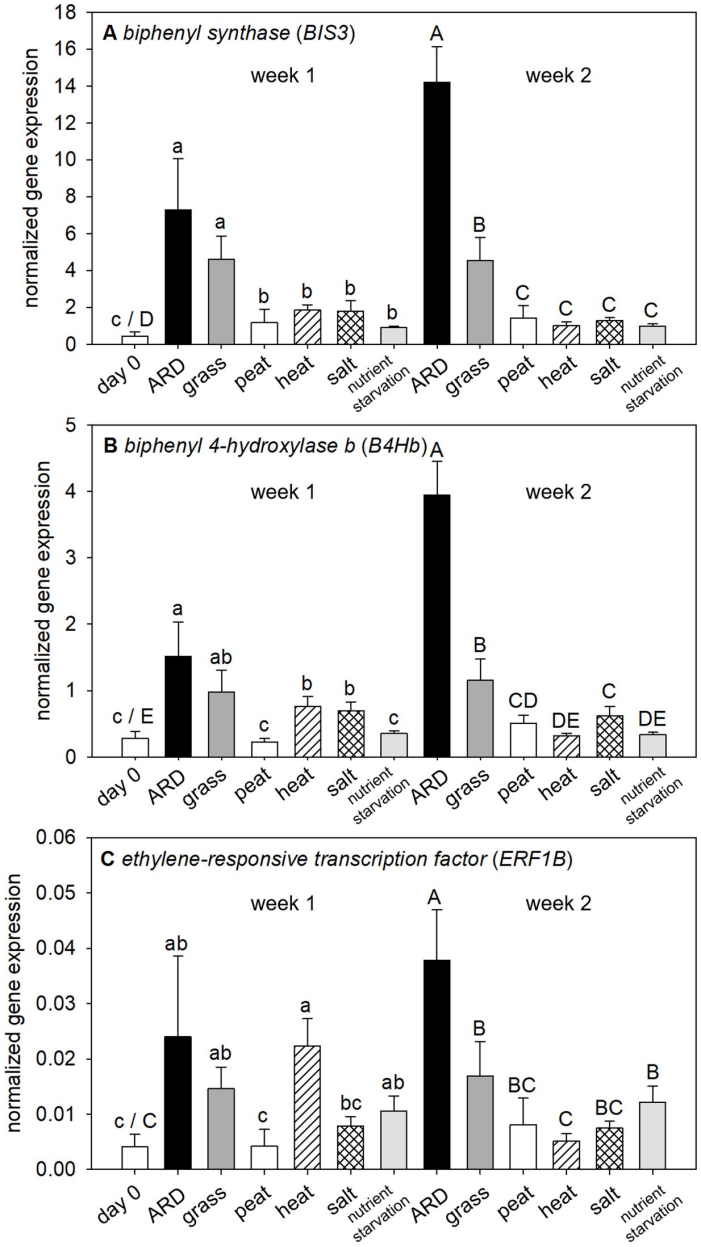

Fig 6. Normalized gene expression of the biomarker candidate genes *biphenyl synthase 3* (*BIS3*) and *biphenyl 4-hydroxylase* (*B4Hb*) and *ethylene-responsive transcription factor 1B-like* (*ERF1B*) in roots of M26 plants grown under the conditions ARD, grass soil, peat substrate, heat, salt stress and nutrient starvation as depicted in Table 1. Means ± SD, n = 5, differences between variants were assessed with a Tukey test for each week ($p < 0.05$). Different letters indicate significant differences between variants for week 1 (small letters) and week 2 (capital letters).

https://doi.org/10.1371/journal.pone.0238876.g006

a clear response to heat stress was observed. At week 1, *ERF1B* gene expression showed a clear peak for the heat stressed plants (Fig 6C), which was not present anymore at week 2, after the plants had been relieved from the stress.

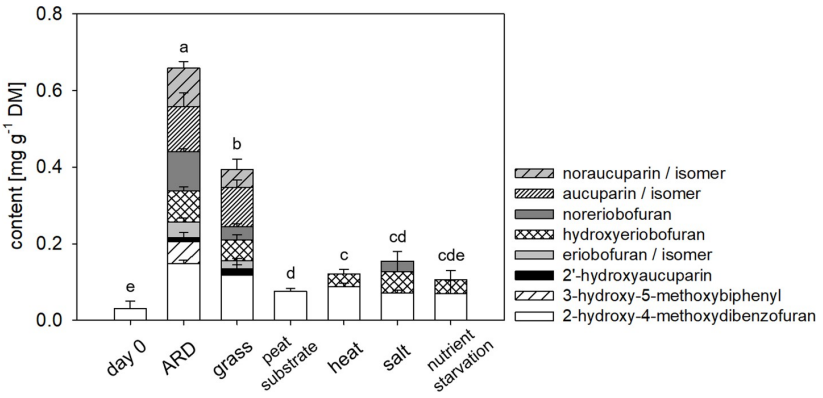

Fig 7. Means of individual phytoalexin compounds measured in M26 roots grown under the conditions ARD, grass soil, peat substrate, heat, salt stress and nutrient starvation after 14 days of culture as depicted in Table 1. Means and standard deviations of 5 plants, except for salt stress (n = 4) are given in the table below.

https://doi.org/10.1371/journal.pone.0238876.g007

Phytoalexin composition was most diverse in the ARD variant compared to other stressors

The total phytoalexin content in roots from experiment 2 after two weeks (Fig 7) correlated with not only the expression of the phytoalexin biosynthesis genes *biphenyl synthase 3* (*BIS3*, $p < 0.0001$) and *biphenyl 4-hydroxylase b* (*B4Hb*, $p < 0.0001$) but also with the *ethylene-responsive transcription factor 1B-like* (*ERF1B*, $p < 0.0001$) (Fig 6A and 6B). The highest total phytoalexin content was present in the ARD variant, followed by the grass variant. In addition, the composition of individual phytoalexin compounds was more diverse in the soil variants ARD and grass than in the other variants. 2-Hydroxy-4-methoxydibenzofuran was again the only phytoalexin detected at a base level in all variants (Fig 7). Hydroxyeriobofuran was present in the soil variants ARD and grass and in the abiotic stress variants heat, salt and nutrient starvation. Furthermore, the salt variant contained an additional small amount of noreriobofuran (Fig 7), which was significantly smaller compared to the ARD and grass variants. The peat substrate did not induce any new phytoalexin. Roots grown in grass soil contained the same profile of phytoalexins as the ARD variant with only 3-hydroxy-5-methoxybiphenyl being exclusively present in the ARD variant. Although noraucuparin was detected in both ARD and grass variants, it was only significantly induced in the ARD variant as well (Fig 7).

The detected content of all phytoalexin compounds, except for 2'hydroxyaucuparin and hydroxyeriobofuran, strongly correlated ($p = < 0.0001$) with the gene expression levels of *BIS3*, *B4Hb* and *ERF1B* (see Table 5). The abundance of single phytoalexin compounds was correlated with shoot and root dry mass, e.g. for noraucuparin (shoot $p < 0.05$, root $p < 0.05$), eribofuran (shoot $p < 0.05$, root $p < 0.05$), noreriobofuran (shoot $p < 0.05$, root $p < 0.05$) and hydroxyeriobofuran (shoot $p < 0.05$, root $p < 0.001$).

Discussion

The experiments conducted in this study aimed at the identification and validation of universal and early biomarkers for ARD responses within the apple roots on the gene expression level. The first experiment compared the reaction of M26 apple plants growing in soils from three ARD sites to identify genes that are universally expressed under ARD conditions. The second experiment investigated the responses of three selected biomarkers to different abiotic stressors to exclude general stress response genes.

Table 5. Results of all-pairs interaction contrast analysis using sandwich estimator of individual phytoalexin compounds measured in M26 roots grown under the conditions ARD, grass soil, peat substrate, heat, salt stress and nutrient starvation after 14 days of culture (Table 1, Fig 7). Different letters indicate significant differences in content as calculated by multiple comparisons (all-pairs interaction contrast analysis using sandwich estimator or nonparametric multiple comparisons for relative contrast effects, $p < 0.05$).

Phytoalexin compound	day 0	ARD	grass	peat substrate	heat	salt	nutrient starvation
2-hydroxy-4-methoxydibenzofuran	d	a	ab	bc	bc	c	bcd
3-hydroxy-5-methoxybiphenyl	b	a	b	b	b	b	b
2'-hydroxyaucuparin	a	a	a	a	a	a	a
eriobofuran	b	a	a	b	b	b	b
hydroxeriobofuran	c	a	ab	c	b	abc	bc
noreriobofuran	c	a	b	c	c	bc	c
aucuparin	b	a	a	b	b	b	b
noraucuparin	b	a	b	b	b	b	b

https://doi.org/10.1371/journal.pone.0238876.t005

Differences in shoot growth alone may not be suitable to assess ARD severity in a soil

With ARD coming to the awareness of fruit and tree producers, methods of identifying and quantifying the disease in the soil are more than ever of interest. Yim et al. [10] developed a bio-test by means of linking the shoot length reduction of the highly ARD-susceptible rootstock M26 in ARD-affected soils compared to the respective disinfected soils as an indication for the presence of ARD. We used this bio-test to compare the reaction of M26 when grown in soil from three ARD sites in a greenhouse experiment. At these sites (Ellerhoop, Heidgraben and Ruthe), ARD had been intentionally induced by repeatedly replanting the apple rootstock 'Bittenfelder Sämling' [12]. The ARD sites share the same cropping history, but differ in their soil structural/physical and chemical parameters and were exposed to different climatic conditions.

After growing in the soils for eight weeks, M26 plants showed typical ARD symptoms in comparison to the respective γ-disinfected controls (Fig 1). The roots of the plants grown in the untreated ARD soil were dark in coloration, which has been reported to be due to cellular damage and necrosis [1, 10, 44] but also deposition of phenolic compounds [10]. In addition, the plants of the untreated ARD variants in all soils had fewer fine roots, which resulted in a significantly reduced root dry mass in ARD soil from Ellerhoop and Heidgraben (Fig 2). A reduction in shoot length and biomass, another major feature of ARD (reviewed in [1]), was observed for soils from Ruthe and Heidgraben (Figs 1 and 2). In the Ellerhoop soil, shoot length and shoot dry mass did not significantly differ between the ARD and γARD variants after eight weeks. This could point to the fact that either chemical or physical characteristics of this soil caused slower growth, beneficial soil organisms were lacking or pathogens survived the irradiation and thus occurred in the γ-irradiated soil. The latter speculation is supported by the observation that roots grown in γ-irradiated Ellerhoop soil contained significantly higher phytoalexin contents compared to the γ-irradiated soils of the other two sites investigated (Fig 4).

The strongest ARD effect in terms of shoot length and fresh mass reduction was found for the sandy soil from Heidgraben (Figs 1 and 2). It has been reported that ARD is easier and faster induced in sandy soils [45]. Ruthe, as a considerably clayey soil, might have had a greater ARD suppressing capacity as reviewed for general disease suppressiveness by Garbeva et al. [45]. The different water holding capacities of the sandy and loamy soil could have also contributed to the observed differences. The lower ARD incidence in this experiment in Ruthe as well as Ellerhoop soil may also be due to waterlogging after heavy rain falls at the experimental

sites. Anaerobic conditions in the soil caused by waterlogging may have an effect similar to other disinfecting measures leading to a weakening of the ARD effect [28, 46, 47].

With only information on shoot length differences from the bio-test, the Ellerhoop soil would have been classified as not or only weakly affected by ARD. It is likely that factors apart from ARD influenced plant growth in this variant. As the precise cropping history of the site Ellerhoop is known, the presence of a replant situation can here be confirmed. However, if the precise history is unknown, as it is the case for most commercial sites, shoot growth reduction alone might not be sufficient to test for the presence of ARD. Other typical ARD symptoms could be observed in the Ellerhoop ARD variants such as root discoloration and reduction in fine roots. A microscopic evaluation of root symptoms has been proposed as a measure to evaluate the presence and severity of ARD early on [44]. An advantage of this analysis is an early-on diagnosis, but routine and experience are required for a microscopic evaluation and the analysis of a large number of samples is time consuming. Therefore, alternative measures for early ARD diagnosis are needed and were identified in the marker genes described below.

BIS3 and *B4Hb* are promising candidates for early ARD indication

Finding an early indicator to assist in the diagnosis of ARD is of major importance. The same importance is attributed to a better understanding of the molecular etiology of ARD in order to deduce effective strategies against it, as the present counteractions present only temporal solutions and are uneconomic and not sustainable (reviewed in [9]). The selection of differently regulated genes from Weiß et al. [11, 33] was narrowed down by testing their expression on further ARD soils in this study. *ACO1*, *GASA1* and *GATAD10* did not show the same difference in regulation between the untreated and γARD variants as reported by Weiß et al. [33], although there was a slight consensus in the expression patterns over time (S1 Fig). The discrepancy between the results obtained in this experiment and the results from Weiß et al. [11, 33] may be caused by other environmental factors during the experiment, which could have triggered the regulation of these genes. As experiment 1 took place in winter (February and March 2017) and Weiß et al. conducted their experiment in March 2014 [11] and September 2014 [33], differences in light and temperature as well as pathogen pressure due to the time of year are expected. Further differences in plant growth and molecular response are anticipated due to the usage of different soils, connected with different cropping histories and inherent microbiome.

In contrast, the remaining three genes, *ERF1B*, *BIS3* and *B4Hb*, are promising candidates for a linkage with ARD and therefore as potential biomarkers for ARD. Taken the presented results into account, the expression of these genes has been shown to differ in response to ARD in three different experiments and in five ARD soils of different origin (this study and [11, 33]). Analyzing these three candidate genes in apple roots in response to the abiotic stressors heat and salt stress and nutrient starvation yielded different specificities, which are presented in detail below.

Expression of the *ERF1B* transcription factor was lower than that of the enzyme-coding genes *BIS 3* and *B4Hb*, resulting in a higher variation in qPCR measurements. Its expression roughly followed the phytoalexin biosynthesis genes with the exception of a clear induction upon heat stress (Fig 6C). At week 2, expression in the heat treated plants resumed to a level comparable to the peat substrate without additional stress. The heat stress was removed after the week 1 sampling time point, indicating that the heat stress induction of *ERF1B* gene expression was temporarily limited to the incidence of stress and declined after that.

Ethylene response factors (ERF) are integral components of ethylene signaling and response. They play a major regulatory role in the molecular response to biotic stressors. Upon

activation, they bind to GCC box elements [48]. The ERF1B transcription factor may link plant hormone signaling pathways of ethylene, jasmonic acid and salicylic acid [49, 50] and is involved in biotic stress responses [49, 51, 52]. Constitutive expression of *ERF1B* has been reported to increase resistance against the necrotrophic fungi *Botrytis cinerea* and *Plectosphaerella cucumerina* in *Arabidopsis thaliana* [49]. In our study, *ERF1B* expression showed a clear induction in ARD soil (Figs 3 and 6C). *ERF1B* expression can be related to the presence of biotic agents in the soil, which are the main causal agents of ARD [5, 10, 53]. As demonstrated in this study, *ERF1B* expression in apple was additionally linked to heat stress. ERF transcription factors are next to biotic stress responses heavily involved in plant responses to abiotic stresses via binding to dehydration-response elements [54]. Abiotic stresses that induce ERF transcription factor expression include salt stress, drought, cold, heat and changes in light availability (reviewed by [48]). In apple, especially drought and salt stress responses are co-regulated [55, 56]. However, an expression induction of this specific *ERF1B* upon salt stress was not observed in our study (Fig 6C).

BIS3 and *B4Hb* are coding for enzymes catalyzing subsequent steps in phytoalexin biosynthesis [33]. Phytoalexins are part of the biotic stress response and have been described in members of the *Rosaceae* including *Malus*, *Pyrus* and *Sorbus* [34, 57, 58]. Evidence of their presence in apple roots was first reported by Weiß et al. [11, 33] and in this study. Expression of *BIS3* and *B4Hb* followed a similar expression pattern upon exposure to ARD (Fig 3). They showed an early significant increase on all soils tested as early as three days after the plants were introduced to the ARD soils, with *B4Hb* in Ellerhoop soil as the only exception. Evaluating *BIS3* and *B4Hb* responses to abiotic stressors indicated that they were outstandingly highly expressed in roots of the ARD variant (Fig 6A and 6B). In addition, they were more highly expressed in the grass soil variant (Fig 6A and 6B). This unspecific response is important to consider regarding the limitations of the explanatory power as expression markers but may also give further insights into the ARD etiology as discussed below. The other stresses investigated did not lead to significant induction of these phytoalexin biosynthesis genes. In the literature, phytoalexins are described to be a part of the induced defense response against biotic stressors, fungi in particular [59]. *BIS3* expression was reported previously in apple upon fire blight infection [60] and upon infection with *Pythium ultimum*, one causal agent of ARD [61, 62]. Taking these studies together with further studies from our group [11, 32, 33, 63], the phytoalexin biosynthesis is a clear and highly specific part of the *Malus* response to ARD.

The expression of *BIS3* and *B4Hb* strongly correlated with the abundance of phytoalexin compounds. The maximum total phytoalexin amounts found in this study, reaching up to 1.08 mg g^{-1} dry mass in the ARD variant of Heidgraben in experiment 1 (Fig 4) were similar to maximum amounts of 1.7 mg g^{-1} dry mass after 14 days reported by Weiß et al. [33]. The authors hypothesized about a possible autotoxic effect of the phytoalexin compounds [33], because comparable concentrations of the phytoalexin compounds camalexin and phaseolin were shown to be cytotoxic to *Arabidopsis thaliana*, *Phaseolus vulgaris* and *Beta vulgaris* cells, respectively [64, 65]. In the second experiment, where phytoalexins were detected after 14 days, the measured contents were lower. The highest total content was 0.66 mg g^{-1} dry mass in the ARD variant (Fig 7). One explanation may be the shorter exposure of the plants to ARD in experiment 2 (14 days) compared to experiment 1 (56 days). However, the amounts detected in the study of Weiß et al. [33] were considerably larger after 14 days. This may indicate further factors influencing the total amount of phytoalexins produced.

Individual phytoalexin compounds differed considerably between variants of both experiments. In experiments 1 and 2, 2-hydroxy-4-methoxydibenzofuran was present in all variants and contributed to the total phytoalexin amount detected in the γARD variants (Fig 4) and considerably to the stress variants (Fig 7). Thus, 2-hydroxy-4-methoxydibenzofuran may not

only be considered as a phytoalexin in apple roots, which by definition is synthesized de novo as a (biotic) stress response. As this compound is already present in the plant before stress application, it may be referred to as phytoanticipin, as suggested by van Etten [66].

The phytoanticipin/phytoalexin 2-hydroxy-4-methoxydibenzofuran was present in all the tested variants and contributed considerably to the total phytoalexin amount detected in the γARD variants (Fig 4) and stress variants (Fig 7). When the apple plants were grown in ARD soil, more phytoalexins including four biphenyls and three dibenzofurans were induced. These changes in the phytoalexin profiles were strongly correlated with the expression pattern of the phytoalexin biosynthetic genes (Figs 3 and 6). Of particular interest is 3-hydroxy-5-methoxybiphenyl, which was only detected in the ARD variant of experiment 2 and at day 10 of experiment 1 (S2 Fig), but was absent in all abiotic stresses as well as in grass soil (Fig 7). Apart from 3-hydroxy-5-methoxybiphenyl, the phytoalexin composition of the ARD and grass soils was almost identical. BIS3 is located at the beginning of the biphenyl biosynthesis pathway, while B4Hb converts 3-hydroxy-5-methoxybiphenyl to noraucuparin [33]. The accumulation of 3-hydroxy-5-methoxybiphenyl at earlier time points of ARD indicates a stronger or faster induction of BIS3 than of B4Hb in ARD soil. In contrast, the induction of BIS3 and B4Hb in grass soil was weaker and equal, therefore no accumulation of 3-hydroxy-5-methoxybiphenyl was detected. To improve our understanding of ARD, the role of particularly 3-hydroxy-5-methoxybiphenyl and the enzyme catalyzing its biosynthesis, caffeic acid 3-O-methyltransferase (SaOMT1), may be of interest. In apple, it was shown that *OMTa* expression showed a significant increase in its transcript level, following the same pattern as *BIS3* and *B4Hb* [33].

The nutrient starvation variant showed the smallest contents in all nutrients, especially of N, P and K. An exception was calcium, which had twice the contents than the other variants. Thus, in this nutrient starvation variant, the visual symptoms of nutrient deficiency are proved. Demidchik et al. [67] showed that a decrease of extracellular K leads to a hyperpolarization of the epidermal plasma membrane followed by an increase of cytosolic Ca in *Arabidopsis thaliana*. This would explain the high calcium content in shoot material of the nutrient starvation variant. Based on the gene expression study and phytoalexin concentration inside the root, it can be concluded that abiotic stress has no effect on phytoalexin synthesis and only biotic factors affect the expression of these biomarker genes.

Further studies including soils from more sites should follow to shed additional light on the quantitative correlation between ARD severity and candidate gene expression.

Buildup of the phytoalexin defense reaction as possible cause of ARD

Results from both experiments presented in this study indicate a time effect in candidate gene expression, with gene expression increasing in the ARD variants (Figs 3 and 6 and S3 Fig). Weiß et al. [33] investigated gene expression in apple roots grown 3, 7, 10 and 14 days in ARD versus γARD soil and observed patterns similar to those we report in this study. With the comparison to grass soil, we have now proven that pathogens present in this control soil also induce the expression of our candidate genes *BIS3*, *B4Hb* and *ERF1B* to a certain extent (Fig 6). Candidate gene expression in ARD soil was complemented by the phytoalexin analysis. After one week, the expression of all three genes in grass soil was nearly comparable to the one in ARD soil, but after two weeks there was a significantly higher expression in ARD soil. Previous studies also reported growth reduction and browning of roots in grass soil in comparison to γ-irradiated grass or ARD soil [12, 44]. Thus, we assume a molecular defense reaction against biotic stress to be also initiated in grass soil, but in ARD soil it was becoming much stronger with time.

ARD develops in healthy soil and consequently an interaction of the plant with the soil leads to the formation of a replant situation. This plant-soil-interaction must be quite unique

to *Malus* and closely-related rosaceous species, as ARD is not affecting non-related species outside the family. Winkelmann et al. [9] proposed the cause of ARD to be harmful shifts in soil microbiota (dysbiosis) regarding their structure as well as their functions. Radl et al. [68] attributed these harmful shifts to the previous culture of the same or closely-related species. As biphenyls and dibenzofurans are defense compounds specific to the subtribe *Malinae* and produced in lower amounts in healthy soils, one could speculate on their involvement in the shift of soil microbiota leading to the development of ARD. This hypothesis would require ARD agents to survive or even utilize these defense compounds where other microorganisms cannot. Further studies are required to test this hypothesis. One of the promising strategies to prove this hypothesis could be the isolation and identification of microbes from ARD soils and then to investigate the effects of biphenyls and dibenzofuran phytoalexin on these isolated microbes. In case the phytoalexins could serve as carbon source to support or even promote the growth of specific microbes, the increased microbes will further induce the phytoalexin production in roots, which could then cause the damage of root cells by cytotoxic properties of phytoalexins. However, this hypothesis needs to be addressed in future research.

Conclusions

In the present study, we identified the expression of the phytoalexin biosynthesis genes *BIS3* and *B4Hb* as suitable biomarkers for apple replant disease (ARD). Their expression was strongly and consistently induced in roots of apple plants grown in three different soils affected by ARD compared to γ-irradiated ARD soil. Furthermore, their expression was not influenced by common abiotic stresses. The expression of *BIS3* and *B4Hb* was strongly correlated with the abundance of phytoalexin compounds. Hence, due to their high expression levels and their high specificity, *BIS3* and *B4Hb* can be recommended as early ARD markers. Future studies should include expression analyses of the ARD marker genes after inoculation with causal agents that are part of the ARD complex. Furthermore, it would be interesting to correlate gene expression levels to the microbial community composition in the respective ARD soils. In ARD soil, phytoalexin biosynthesis was increased in comparison to healthy soil. The presence of these apple-specific defensive compounds in healthy soil led to the hypothesis that they might play a role in the ARD etiology by attracting specific communities of soil-borne pathogens.

Supporting information

S1 Fig. Normalized gene expression of *1-aminocyclopropane-1-carboxylate oxidase homolog 1-like* (*ACO1*), *gibberellin-regulated protein 1-like* (*GASA1*) and *GATA zinc finger domain-containing protein 10-like* (*GATAD10*) in M26 roots growing in ARD and γARD soil from the sites Ellerhoop, Heidgraben, and Ruthe. Means ± SD, n = 4 pooled samples. Significant differences at each time point shown for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) as investigated by Tukey tests.
(PNG)

S2 Fig. Means and standard deviations of individual phytoalexin compounds measured in M26 roots grown in either ARD or γARD soils from the sites Ellerhoop, Heidgraben and Ruthe after 10 and 56 days. Number of root samples analyzed indicated by n.
(PNG)

S3 Fig. Means and standard deviations of summarized phytoalexin compounds measured in M26 roots grown in either ARD or γARD soils from the sites Ellerhoop, Heidgraben

and Ruthe over a course of 56 days. Number of samples analyzed is indicated in S4 Table.
(PNG)

S4 Fig. Typical leaf and root symptoms of the variants in experiment 2. See Table 1 for details on the variants.
(PNG)

S5 Fig. Shoot length of M26 grown under the conditions ARD, grass soil, peat substrate, heat, salt stress and nutrient starvation (n = 5 individual plants, except nutrient starvation n = 4). Different letters indicate a statistical difference (Tukey Test, $p < 0.05$) between variants. See Table 1 for details on the variants.
(PNG)

S1 Table. Sampling schedule for experiments 1 and 2.
(XLSX)

S2 Table. Information on biological and technical replicates of experiment 1.
(DOCX)

S3 Table. Information on biological and technical replicates of experiment 2.
(DOCX)

S4 Table. Pooling and masses of freeze-dried root material from experiment 1 for phytoalexin analysis.
(DOCX)

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4 Split-root approach reveals localized root responses towards apple replant disease (ARD) in terms of ARD biomarker gene expression and content of phenolic compounds

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Abstract

Apple replant disease (ARD) is a threat to apple fruit and tree production worldwide caused by shifts in the soil microbiome. Chemical and physical measures of soil disinfection are the most employed countermeasures but inter-row plantings demonstrate that ARD is widely immobile in the soil, which causes the plant to face ARD affected and healthy soil simultaneously in the field. We simulated this situation in a split-root approach and investigated the plant's macroscopic and molecular reaction to localize its response. Root growth was severely reduced in two ARD soils but the plants were able to level shoot growth out in part as long as half of the root system was growing in healthy soil. In ARD affected roots, the expression of three ARD biomarker genes, *biphenyl synthase 3*, *biphenyl 4-hydroxylase* and *ethylene-responsive transcription factor 1B-like*, was locally induced after two weeks in comparison to disinfected ARD or control soil. Phenolic compound profiles in the roots were studied after eight weeks and showed a high variation between samples and soils. The total phenolic content as well as the contents of epicatechin and an unknown phenolic compound were locally increased in roots grown in ARD soil in comparison to disinfected ARD soil after eight weeks. Our results show how apple plants focus their molecular reaction towards ARD locally in the roots and are able to partly counteract the negative effects caused by ARD by growing into unaffected soil regions. The local nature of ARD presents both challenges in the assessment of ARD severity in the field and for possible management strategies involving soil tillage.

Keywords: gene expression, *Malus domestica*, quantitative RT-PCR, quantitative RP-HPLC-DAD, soil sickness, polyphenols, systemic response

Abbreviations:

ARD apple replant disease

B4H biphenyl 4-hydroxylase

BIS3 biphenyl synthase 3

EF1a, b elongation factor 1-alpha, -beta

ERF1B ethylene-responsive transcription factor 1B-like

GAE gallic acid equivalent

TAC total antioxidant capacity

TPC total phenolic content

TUBB tubulin beta chain

γARD gamma-irradiated ARD soil

1 Introduction

Apple replant disease (ARD) is a worldwide economic risk in apple fruit and tree production, where apple trees are replanted at the same site (Mazzola and Manici, 2012; Manici et al., 2013; Winkelmann et al., 2019). As a soil-borne disease complex, ARD causes characteristic symptoms of stunted shoot growth, which is linked to root damage below ground (Hoestra, 1968; Yim et al., 2013; Grunewaldt-Stöcker et al., 2019). *Nectriaceae*, *Pythium*, *Rhizoctonia* and actinomycetes (Mazzola, 1997; Mazzola, 1998; Tewoldemedhin et al., 2011b; Mazzola and Manici, 2012; Manici et al., 2018; Radl et al., 2019) have been identified as the main causal agents of ARD with regional differences on a global scale. The current view on the cause of ARD is that shifts in the soil (micro)biome caused by the previous apple culture impede the soil's capability to sustain the following apple culture (Winkelmann et al., 2019). The negative effects of ARD on yield and fruit and tree quality are influenced by numerous biotic and abiotic factors, making the economic losses difficult to calculate (Peterson and Hinman, 1994; Geldart, 1994; Mazzola and Manici, 2012; Manici et al., 2013). ARD is immobile in the soil and therefore, inter-row planting can be a practical option to overcome the disease. However, ARD persists in the soil over many years (Hoestra, 1994), which makes this option viable only once and illustrates the need for a long-term sustainable solution. This is even more urgently needed, since current countermeasures are either not environmentally friendly (soil fumigation) or hardly available (field changes to use virgin soils).

Research on understanding the molecular response of apple towards ARD was undertaken in order to unravel the disease etiology but also to identify early indicators of ARD on a molecular level. These indicators may be useful as biomarkers aiding the early diagnosis of ARD, but also for identifying tolerance or sensitivity features which may be in turn useful in the breeding of ARD tolerant rootstocks. The apple plants strongly induce their defense against biotic stress in the roots when they are facing ARD affected soils (Zhu et al., 2014; Shin et al., 2016; Weiß et

al., 2017a; Weiß et al., 2017b; Weiß and Winkelmann, 2017; Reim et al., 2020; Rohr et al., 2020). Strikingly, the biosynthesis of phytoalexins, Malinae specific phenolic defense compounds, was found to be highly induced in these roots (Weiß et al., 2017b).

Growth in ARD soil causes an antioxidative stress response (Henfrey et al., 2015). The authors detected higher total phenolic contents in roots and a higher antioxidant capacity in leaves of apple seedlings (derived from seeds of the cultivar 'Golden Delicious') after growth on ARD affected soil compared to the control. The elevated antioxidant levels in leaves after growth on ARD soil were responsible to induce a slight tolerance against oxidative stress caused by the use of an herbicide (Henfrey et al., 2015). In addition, roots of apple are known to be rich in phloridzin, the content of which was increased after growth on ARD affected soil (Hofmann et al., 2009). Leisso et al. (2017) identified other phenolic compounds, e.g. benzoic acid, rutin and phloretin, and showed that the phenolic profile of rhizodeposits differ between rootstocks postulated as tolerant or susceptible to ARD. In addition, the relative proportion of phenolic substances differed between root orders and developmental stages of the roots grown in ARD soil (Emmett et al., 2014). These phenolics may also be involved in the development of dark necrotic lesions of infected roots, which are typical ARD symptoms detected by Grunewaldt-Stöcker et al. (2019).

The above-mentioned immobility of ARD in the soil leads to a situation where different parts of the plant’s root system are faced with both healthy and ARD affected soil simultaneously when exploring the soil. In a recent study (Lucas et al., 2018), the spatial distribution of the apple plant’s response to ARD was investigated in a split-root approach. The typical symptoms of root browning and growth reduction as well as impaired nutrient uptake were only observed in the ARD-affected soil compartments indicating a localized response of the plant.

In this study, we used a split-root approach to simulate the situation in the field when the apple plant is faced with healthy and ARD soil simultaneously to investigate molecular reactions

towards ARD in the roots. We conducted two experiments employing ARD and control soils from two sites in order to answer the question, whether the root's response to ARD is local or systemic. Therefore, we analyzed the expression of three ARD biomarker genes (Rohr et al., 2020) *biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase* (*B4H*) and *ethylene-responsive transcription factor 1B-like* (*ERF1B*) in roots of one plant grown in ARD affected soils in one compartment and ARD unaffected soils in the neighboring compartment of the split-root box. Additionally, using this approach, the total phenolic content (TPC), the total antioxidant capacity (TAC) as well as the phenolic spectrum of root extracts were surveyed. Thus, the objectives of this study were (i) to link the previously observed localized growth response to a local response of the selected genes and phenolic metabolites and (ii) identify the phenolic compounds that can be useful in unraveling ARD.

2 Materials and methods

2.1 Soil origins and treatments

For experiment 1, 40 L of ARD soil from the ORDIAmur (www.ordiamur.de) reference site Heidgraben (Baumschule Harald Klei, Heidgraben, 53°41'57.5"N 9°40'59.6"E, Mahnkopp et al., 2018) was sampled at a depth of 5 – 20 cm in October 2017. The soil was sieved by 4 mm and half of the volume was packed in autoclavable bags and sent for disinfection by gamma irradiation at a minimum dosage of 10 kGy (Synergy Health Radeberg GmbH**,** Radeberg, Germany). The soil was stored at 4 °C until the start of the experiment. The water content was 14.8 %, determined by drying subsamples in triplicates for 3 days at 105 °C. Experiment 1 included the soil variants Heidgraben ARD untreated (ARD) and Heidgraben ARD disinfected (γARD) in a total number of 24 chambers (Table 1) and took place from 10th of January to 7th of March 2018.

The soil for experiment 2 was collected correspondingly in August 2018 from the ORDIAmur ARD reference site Ellerhoop (Gartenbauzentrum Schleswig-Holstein, Ellerhoop, 53°42'51.9"N 9°46'13.0"E, Mahnkopp et al., 2018) and treated and stored in the same manner as described above. For Ellerhoop, soil from the ARD plots and adjacent grass plots (representing ARD-unaffected control soil) was sampled, of which half was subjected to gamma irradiation (Table 1). For experiment 2, the water contents were determined after gamma irradiation, which were as follows: 10.6 % Ellerhoop ARD untreated (ARD), 9.8 % Ellerhoop ARD gamma (γARD) and 12.5 % Ellerhoop grass (grass). Experiment 2 included a total number of 28 split-root boxes (Table 1) and took place from 11th of October to 6th of December 2018.

2.2 Experimental design

Both experiments were conducted using split-root boxes made according to the template of Lucas et al. (2018). Each box consisted of two adjacent compartments (each 32.5 x 10 x 2 cm, h x w x d) holding a volume of 0.65 L each. The back of the boxes was equipped with water retention fleece (material: polyester / polypropylene, water holding capacity 2.5 L m^{-2}) separately for each compartment. The fleece was covered with a 30 µm pore diameter gauze (type PES-30/21, Franz Eckert GmbH, Waldkirch, Germany) to prevent roots from growing into the fleece. A spacer was used to evenly fill the compartments while preventing contaminations between the two halves of the box.

Each soil (Table 1) was mixed with 2 g L^{-1} of Osmocote Exact Standard 3-4 M (16 % total nitrogen, 9 % phosphorus pentoxide, 12 % potassium oxide, 2 % magnesium oxide + trace elements, Everris International B.V., Geldermalsen, The Netherlands, https://icl-sf.com/global-en/products/ornamental_horticulture/8840-osmocote-exact-standard-3-4m). Following Lucas

et al. (2018), soil bulk density was adjusted to 1.2 $g_{dry\ soil}$ cm^{-3} according to the respective water contents (see above). At the start of the experiment, the water content was adjusted to 20 % but during the course of the experiment water was added as required. This was necessary since monitoring of the water content by weight did not account for the differences in root growth and subsequently water uptake among the two compartments of one rhizobox.

Table 1: Overview of all rhizobox variants and replicates for sampling in experiments 1 and 2.

Experiment	Soil origin		Substrate combinations in rhizoboxes									
			γARD	γARD	γARD	ARD	ARD	ARD	ARD	Grass	Grass	Grass
1	Heidgraben	Boxes in total	5		14		5		not analyzed		not analyzed	
		Replicates for gene expression after 14 d	3	3	6	6	3	3				
		Replicates for phenolic compound analysis after 56 d	2	2	7	7	2	2				
2	Ellerhoop	Boxes in total	4		8		4		8		4	
		Replicates for gene expression after 14 d	2	2	4	4	2	2	4	4	2	2
		Replicates for phenolic compound analysis after 56 d	2	2	4	4	2	2	4	4	2	2

2.3 Plant material and growth conditions

Clones of the ARD susceptible apple rootstock 'M26' (hereafter termed M26) were propagated in vitro and acclimatized to greenhouse conditions as described in Rohr et al. (2020). Five weeks after acclimatization, the plants were removed from the peat substrate, washed gently and placed into the filled split-root boxes with half of their root system facing each compartment. After planting, the clear front cover was affixed and subsequently covered with aluminum foil (experiment 1) or black plastic foil (experiment 2) to provide darkness for undisturbed root growth. A thin layer (ca. 1.5 cm) of 2-6 mm vermiculite was added on top of

the soil to prevent drying out of the roots sitting directly on top of the barrier between the compartments. The boxes were placed in stands with the windows facing down at a 30° angle (supplementary material 1). This allowed an observation of root growth along the clear front panel.

All chambers were placed in the greenhouse in a randomized design at 19.1 ± 1.8 °C and 69.4 ± 2.7 % relative humidity for experiment 1 and 20.1 ± 2.8 °C and 64.0 ± 9.9 % relative humidity for experiment 2. During the first three days, the plants were cultivated without additional lighting. From then on, 16 h of daylight were provided by SON-T Philips Master Agro 400 W lamps (Hamburg, Germany) whenever solar radiation fell below 25 klx. Plant protection was carried out weekly against thrips with 3 % of the ecological insecticide NeemAzal-T/S (Trifolio-M GmbH, Lahnau, Germany) according to integrated plant protection. In experiment 2, plants showed beginning symptoms of infections with a fungal leaf spot disease. To impede fungal growth, microfine Sulphur was applied in the night hours between weeks 2 and 5 and the relative humidity was decreased from 70 % to 50 % from week 5 on.

2.4 Sampling and data collection

Throughout the duration of the experiments of 8 weeks, shoot length was measured weekly and root growth was documented photographically every second week.

Samples for gene expression analysis were taken after 14 days by gently removing the plants from the chambers whilst keeping the two root halves separated. In single boxes, roots attempted to grow horizontally from one compartment to the adjacent one. These roots were removed and not used for the following analyses. The roots were briefly washed in lukewarm tap water, blotted dry, transferred to 2 mL reaction tubes and shock-frozen in liquid nitrogen. An overview of sample numbers subjected to gene expression analysis is presented in Table 1.

The final evaluations of both experiments took place after 56 days. The plants were removed from the chambers, the root systems were cleaned and the plants were separated into the two root halves and the shoots to determine the fresh masses of each part. For experiment 2, the dry masses were determined as well after freeze-drying the samples for three consecutive days (Christ ALPHA 1- 4 LSC, Osterode, Germany). These samples were subjected to further analyses to determine phenolic compounds and the TAC (see chapter 2.6).

2.5 Gene expression analyses

2.5.1 RNA isolation and first strand cDNA synthesis

Two 4 mm cooled steel beads were added to each reaction tube of the root samples (Table 1). The samples were homogenized for 2:30 min at 27 Hz in a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) under liquid nitrogen cooling. Total RNA was isolated from approximately 100 mg fresh mass using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) according to the manufacturer's instructions with the provided extraction buffer for phenol-containing plant material (RP lysis buffer) and 40 µL of elution buffer. Quantity and quality of the resulting RNA were determined spectrophotometrically (NanoDrop 2000c, Peqlab, Erlangen, Germany) and additionally the quality was assessed on 1 % agarose gels. The samples were digested with DNase I (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions to remove genomic DNA and stored at -80 °C prior to cDNA synthesis.

First strand cDNA synthesis was carried out with an input of 1 µg of RNA and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The procedure was carried out according to the manufacturer's instructions using the provided oligo-dT primers. The resulting cDNA was diluted tenfold and stored in aliquots at -20 °C for RT-qPCR measurements.

2.5.2 Quantitative RT-PCR

All PCR reactions were run on a CFX Connect instrument (Bio-Rad, Hercules, CA, USA) with the SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and final primer concentrations of 20 nM. The ARD biomarker candidate genes *biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase* (*B4H*) and *ethylene-responsive transcription factor 1B-like* (*ERF1B*) had been identified in our previous studies (Rohr et al., 2020). *Elongation factor 1-α* (*EF1a*), *elongation factor 1-β* (*EF1b*) and *tubulin beta chain* (*TUBB*) (Weiß et al., 2017a; Weiß et al., 2017b) were selected as reference genes after their expression stability had been tested. Quantification cycles (c_qs) were recorded with the CFX manager software v. 3.0 and the following protocol: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C plus melt curve analysis (65 °C to 95 °C for 5 s each with an increment of 5 °C). A dilution series (1:5, 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:2000) was created from equal amounts of cDNA of each sample. Amplification efficiencies were then determined by plotting the c_qs of the dilution series against the respective log starting quantity within the CFX manager software according to Pfaffl (2001). All primers are listed with their respective amplification efficiencies in Table 2. Gene expression analysis was performed with 6 (experiment 1) or 4 (experiment 2, see Table 1) biological and two technical replicates. *ERF1B* was measured in three technical replicates due to its low expression level. Normalized gene expression was calculated according to Pfaffl (2001).

Table 2: *Primer sequences and amplicon lengths of candidate and reference genes used in quantitative RT-PCR analyses (Weiß et al., 2017a). MDP ID: Malus domestica predicted gene ID (Velasco et al., 2010). Amplification efficiency in % (E [%]) with corresponding coefficient of correlation (R^2).*

Gene name (MDP ID)	Abbreviation	Primer sequence 5' – 3'	Amplicon length [bp]	E [%]	R^2
***ethylene-responsive transcription factor 1B-like* (MDP0000127134)**	*ERF1B*	f: GTCACCTGAATCTTCGTTTG r: GGAAATCAGACCGTAGAGAAG	121	93.1	0.990
***biphenyl synthase 3* (MDP0000287919)**	*BIS3*	f: GGCAAGAAGCAGCATTGAAAG r: CACAACCTGGCATGTCAAC	105	95.7	0.999
***biphenyl 4-hydroxylase* (MDP0000205306, MDP0000152900)**	*B4H*	f: GCTGAGTATGGCCCGTATTG r: AGGAACCCGTCGATTATTGG	156	94.7	0.997
***elongation factor 1-alpha* (MDP0000304140)**	*EF1a*	f: GAACGGAGATGCTGGTATGG r: CCAGTTGGCTCCTTCTTCTC	159	91.8	0.999
***elongation factor 1-beta 2-like* (MDP0000903484)**	*EF1b*	f: GAGAGTGGGAAATCCTCTG r: ACCAACAGCAACCAATTTC	138	96.4	0.996
***tubulin beta chain* (MDP0000951799)**	*TUBB*	f: TTCTCTGGGAGGAGGTACTG r: GTCGCATTGTAAGGCTCAAC	147	92.6	0.993

2.6 Analyses of phenolic compounds and TAC

2.6.1. Extraction method

One hundred mg of roots per sample (experiment 1: fresh roots, exp. 2: freeze-dried roots) were shock frozen with liquid nitrogen and ground in a ball mill (Retsch, Haan, Germany). Pulverized roots were then transferred with 3 mL methanol (exp. 1) and 2 mL methanol (exp. 2) into centrifuge tubes, and centrifuged at 4.700 rpm for 3 min at 3 °C. The supernatant was removed into a volumetric flask, filled up to 5 mL (exp. 1) and up to 2 mL (exp. 2) with methanol before filtration through a 0.45 µm syringe filter. Extracts of experiment 1 were divided into three aliquots. If necessary, samples were then diluted with methanol (1:10 or 1:20)

to ensure quantification of the parameters to be analyzed. Samples were stored at -20 °C until analysis.

2.6.2 Total antioxidant capacity (TAC)

For TAC determination the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, ABTS) method described by Erel (2004) was used modified in house by Havelt et al. (2019). Briefly, 2 mL acetate buffer (0.4 M, pH 5.8, 1 g L^{-1} Tween 80) were mixed with 50 µL extract (blank: 50 µL methanol). The reaction was started by adding 200 µL of ABTS*+-solution (10 mmol L^{-1}, pH 3.6). Each assay contained at least one blank. The absorption was measured at λ = 660 nm (Lambda 25 UV/VIS Spectrometer, Perkin Elmer, Waltham, MA, USA) after exact 5 minutes of incubation. The TAC was calculated by external calibration with Trolox and expressed in mg g^{-1} Trolox equivalent (TE).

2.6.3 Total phenolic content (TPC)

The total phenolic content (TPC) in methanolic root extracts was determined by a method described in Schmitz and Noga (2000) and Havelt et al. (2019). 250 µL methanolic root extract (blank: 250 µL methanol; at least one methanolic blank per assay) were pipetted to 250 µL deionized water into centrifuge tubes. The addition of 250 µL Folin-Ciocalteau reagent started the reaction. After exaction for 30 seconds, 2.5 mL of aqueous sodium hydroxide (0.1 %) were added. The absorption of each sample was measured at λ = 720 nm (Lambda 25 UV/VIS Spectrometer) after exactly 30 minutes. Gallic acid was used as an external standard and results were expressed in mg g^{-1} gallic acid equivalent (GAE).

2.6.4 Quantification of phenolic compounds by HPLC-DAD

For qualification and semi-quantification of single phenolic compounds in methanolic root extracts of experiment 1, a HPLC-DAD system (Agilent, USA, 1200 series) was used equipped with a reversed phase column (LiChroCART, C-18 endcapped, 5 µm; 4.6 x 150 mm). Column temperature was held constant at 25 °C during analysis. Solvent flow was 1 mL min^{-1} and

solvents were (A) water/acetic acid (0.1 %) and (B) acetonitrile. A gradient system was chosen to analyze 10 µL of methanolic extract: 0-10 min ramping from 100 % A to 90 % A, 10-30 min to 70 % A, and back to 90 % A at 33 min. Identification of single phenolic substances took place at λ 254.4 nm (quercitrin), 280.4 nm (epicatechin and phloridzin), 320.4 nm and 370.4 nm with a reference wavelength of 360.4 nm. External standard solutions of epicatechin, quercitrin and phloridzin (each c = 1 mg L^{-1}) were injected to determine their retention time and peak area for semi-quantification.

Due to methodological advancements, the method was changed in 2020 and a HPLC-DAD system (Agilent, USA, 1260 series) with a reversed phase column (Multosphere 120 RP 18 HP, 4.6 x 150 mm) was used for separation and quantification of phenolic compounds. Column temperature was held constant at 22 °C during analysis. Solvent flow was 1 mL min^{-1} and solvents were (A) water/acetic acid (0.1 %) and (B) acetonitrile. A gradient system was chosen to analyze 5 µL of methanolic extract: 0-5 min ramping from 95 % A to 90 % A, holding 90 % A till 20 min, 20-28 min ramping to 80 % A, 28-37 min to 75 % A, 37-51 min to 50 % A, 51-53 min to 95 % A and holding 95 % A till 59 min to re-equilibrate the column. Identification of single phenolic substances took place at wavelength 254.4 nm (rutin and quercitrin), 260.4 nm (3,4-dihydroxybenzoic acid), 280.4 nm (catechin, epicatechin, cinnamic acid, phloridzin and phloretin), 300.4 nm (coumaric acid) and 320.4 nm (chlorogenic acid, caffeic acid and ferulic acid). All substances were calibrated by an external standard calibration in a range of 0.0005 - 0.02 g L^{-1} (Phloridzin: 0.075 - 3.0 g L^{-1}). All compounds were quantified at λ = 280.4 nm.

2.7 Statistical analyses

All data were evaluated using R version 3.6.3 in RStudio 1.2.5033 (R Development Core Team, 2018). Normal distribution was checked and if necessary, data were log transformed prior to

the statistical analysis. For each dataset, a linear model was adjusted and an analysis of variance (ANOVA) was calculated. For the shoot length datasets of both experiments, the statistical evaluation was carried out for every time point separately. Multiple comparisons were calculated with a Tukey test using the R package 'multcomp' version 1.4-12 (Hothorn et al., 2008) with $p < 0.05$. A Spearman correlation was calculated using the 'Hmisc' R package (version 4.4-0, Harrell, 2020) and visualized using 'corrplot' (version 0.84, Wei and Simko, 2017).

3 Results

The split-root setup was chosen as an experimental approach in this study to investigate the local response of roots to ARD soil in terms of growth, candidate gene expression and phenolic compound profiles. Therefore, the mixed variants were of utmost interest, in which roots of one plant were simultaneously exposed to ARD affected soils in one compartment of the rhizoboxes and ARD-unaffected soils (γARD or grass) in the neighboring compartment.

3.1 Shoot growth

Over the eight weeks of the first experiment, plants showed a lag phase of both shoot and root growth in the split-root system. Plants grown in the ARD/ARD rhizoboxes showed strongly stunted shoot growth, which became even more pronounced over the course of the experiment. Between weeks 4 and 5 in both experiments, a rapid increase in shoot growth occurred, leading to a significant difference in shoot length and fresh mass between the γARD/γARD (7.36 ± 0.65 g) and ARD/ARD (1.17 ± 0.64 g) rhizoboxes at the end of experiment 1 (Figures 1, 2, S1 and S2). Plants grown in the mixed γARD/ARD rhizoboxes had an intermediate shoot fresh mass of 4.50 ± 1.57 g, which did not differ significantly from the γARD/γARD variant.

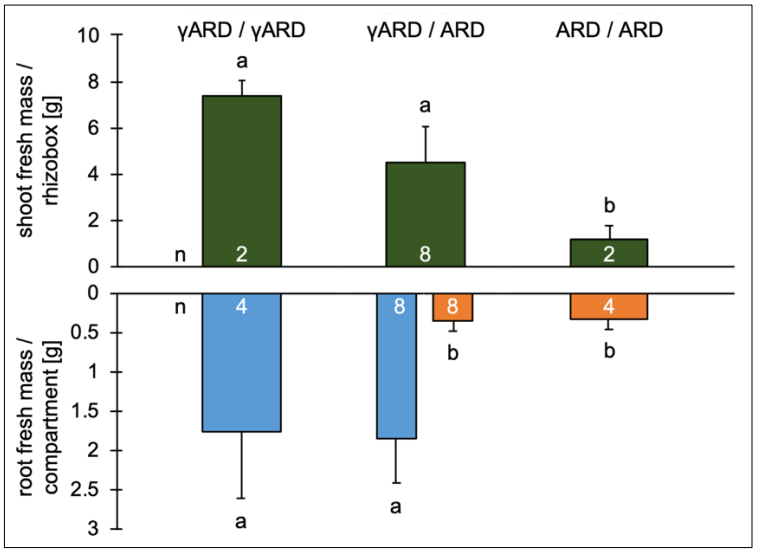

Figure 1: Shoot and root fresh masses and standard deviations of experiment 1 grown in split-root chambers filled with γ-disinfected (γARD) or untreated ARD soil (ARD) for 8 weeks. different letters between variants indicate significant differences (Tukey test, $p < 0.05$, n = individual shoots or roots halves as indicated in the figure).

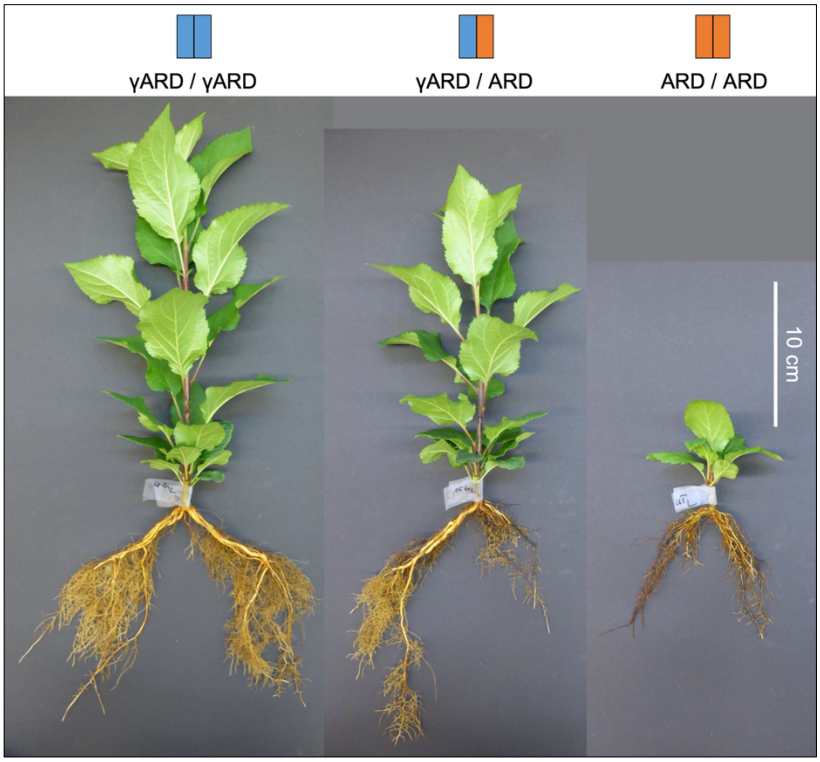

Figure 2: Representative plants of experiment 1 grown in split-root chambers filled with γ-disinfected (γARD) or untreated ARD soil (ARD) for 8 weeks.

For experiment 2, the differences were less pronounced, but again after 8 weeks the most vigorous shoot growth was recorded in the γARD/γARD variant (shoot fresh mass 13.15 ± 1.09 g, shoot length 31.2 ± 3.3 cm), followed this time by the grass/grass variant (8.67 ± 3.04 g shoot fresh mass, shoot length 20.6 ± 7.2 cm, Figures 3, S3). In contrast to experiment 1, plants of the mixed rhizoboxes γARD/ARD (fresh mass 7.60 ± 1.92 g, shoot length 18.2 ± 4.8 cm) and ARD/grass (fresh mass 5.45 ± 1.56 g, shoot length 16.6 ± 1.7 cm) had a significant lower shoot fresh mass and shoot length than those in γARD/γARD rhizoboxes and comparable to the ARD/ARD variant (fresh mass 6.25 ± 0.34 g, shoot length 12.9 ± 1.6 cm) at the end of the experiment (Figures 3, S3, S4).

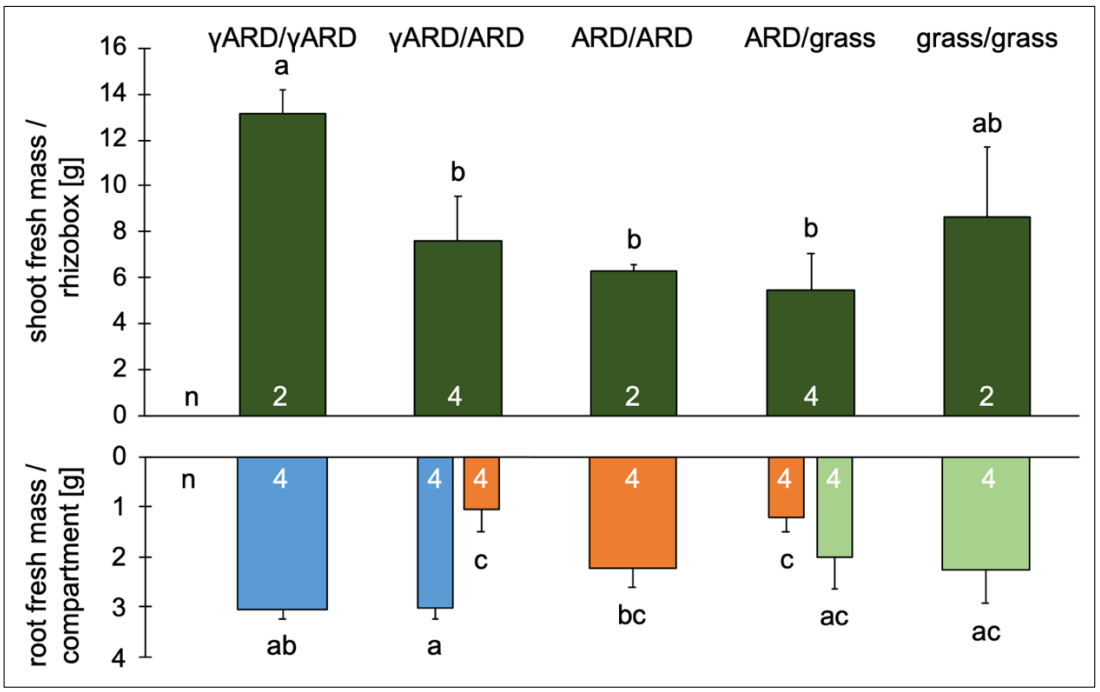

Figure 3: Shoot and root fresh masses and standard deviations of experiment 2 grown in split-root chambers filled with γ-disinfected ARD soil (γARD), untreated ARD soil (ARD) or grass soil (grass) for 8 weeks. Different letters between variants indicate significant differences (Tukey test, $p < 0.05$, n = individual shoots or roots halves as indicated in the figure).

3.2 Root growth

The rhizoboxes allowed the observation of root growth over the course of the whole experiment with a focus on the split-root variants with different soils in the two compartments, i.e. γARD/ARD and ARD/grass. Between weeks 1 and 2 new roots started to emerge. Already at the sampling of the roots for gene expression analysis after 14 days, the beginning of the typical browning of the roots in contact with ARD soil was observed in both experiments (Figures S5, S6). For plants with only half of their root system exposed to ARD soil, only this respective part showed these ARD symptoms, whereas the roots grown in the neighboring compartment with grass or γARD soil appeared more healthy (Figure 4). After 8 weeks, roots grown in the γARD/γARD rhizoboxes had healthy, well branched roots, while roots grown in the ARD/ARD variants were considerably smaller and darker in coloration (Figure 4). Interestingly, this effect was also seen locally for the ARD compartments of the mixed rhizoboxes but not in the γARD compartments (γARD/ARD, Figure 4). In experiment 1, root fresh mass differed significantly between the γARD/γARD and the ARD/ARD variants, reaching a respective average of 1.76 ± 0.86 g and 0.33 ± 0.13 g after 8 weeks (Figure 1). In the mixed γARD/ARD rhizoboxes, root biomass differed significantly between the two compartments: Roots in the γARD compartment reached an average fresh mass of 1.85 ± 0.57 g, while those in the ARD compartment reached 0.34 ± 0.15 g (Figure 1).

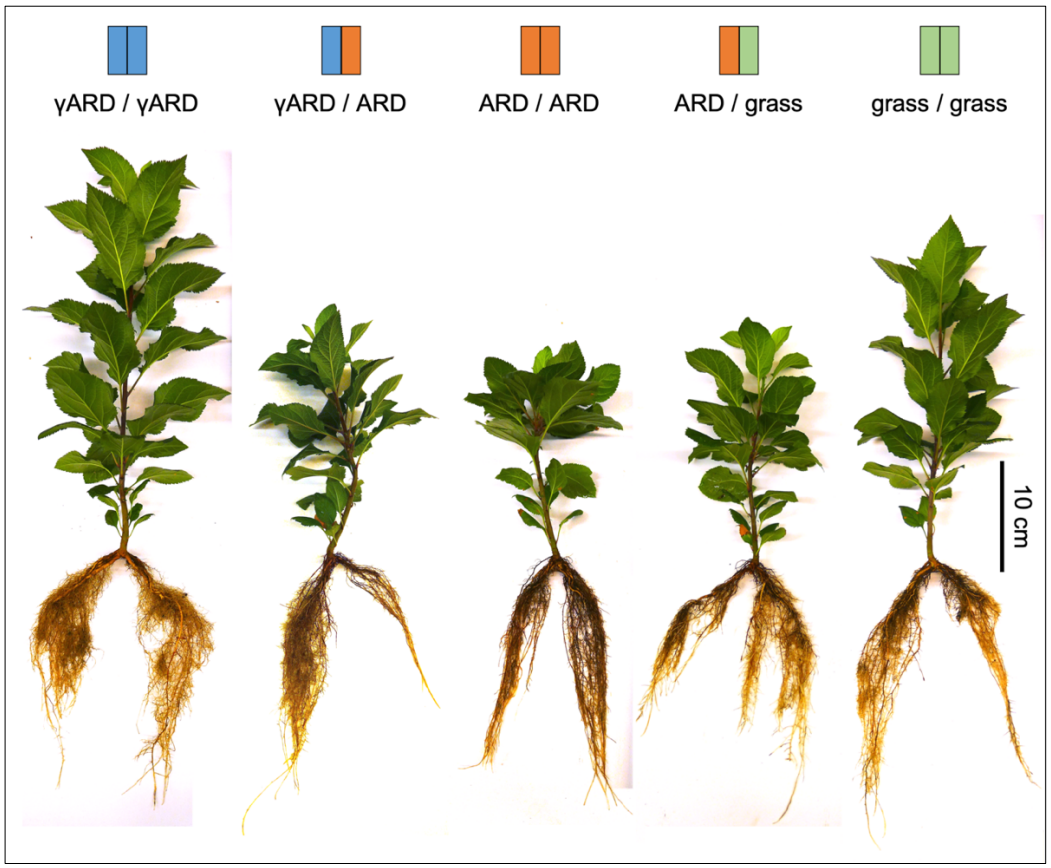

Figure 2: Representative plants of experiment 2 grown in split-root chambers filled with γ-disinfected ARD soil (γARD), untreated ARD soil (ARD) or grass soil (grass) for 8 weeks.

In experiment 2, browning of the roots showed the strongest extent in the ARD/ARD rhizoboxes but was also observed to considerable degree in the grass/grass chambers (Figure 5). This was reflected in the root fresh biomasses (Figure 3). Overall, higher root biomasses were achieved in experiment 2 when compared to experiment 1, ranging from 3.05 ± 0.19 g in γARD/γARD to 1.94 ± 0.35 g in ARD/ARD rhizoboxes. In tendency, roots grown in the ARD compartments of the γARD/ARD and ARD/grass rhizoboxes possessed even a lower biomass than those grown in the ARD/ARD rhizoboxes.

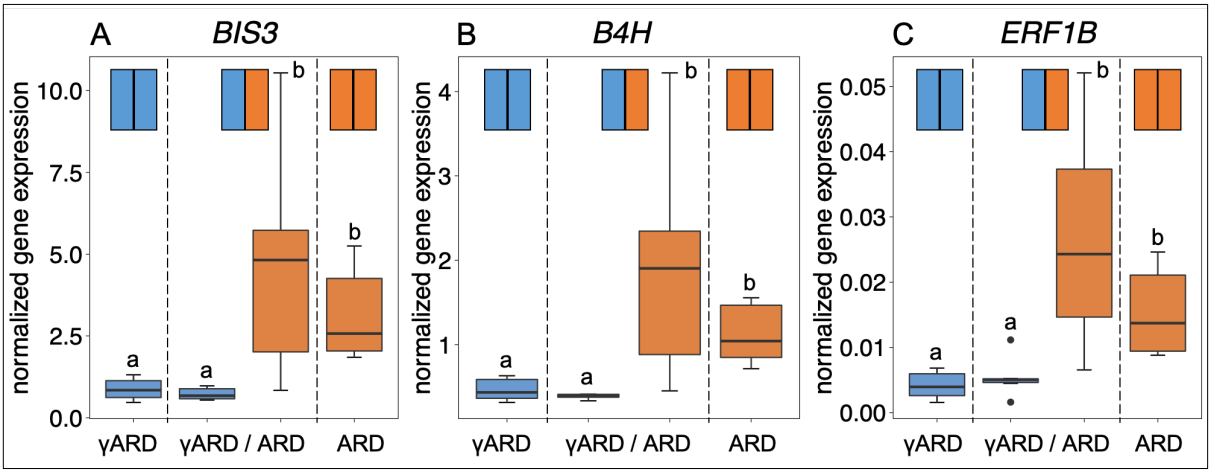

Figure 3: Normalized expression of the ARD candidate genes in experiment 1 analyzed after 2 weeks of culture in split-root chambers filled with γ-disinfected (γARD) or untreated ARD soil (ARD). *Biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase* (*B4H*), *ethylene-responsive transcription factor 1B* (*ERF1B*). Different letters between variants indicate significant differences (Tukey test, $p < 0.05$, $n = 6$).

3.3 Expression of ARD marker genes after 14 days

The expression of the three ARD biomarker candidate genes *BIS3, B4H* and *ERF1B* was analyzed in roots sampled after two weeks, where ARD symptoms of shoot stunting were starting to become distinguishable (Figures S4, S5). The expression of all three genes differed between the γARD/γARD and ARD/ARD rhizoboxes of both experiments with a significantly higher expression in the ARD/ARD variant (Figures 5, 6). In the γARD/ARD chambers, the expression of all three candidate genes differed significantly between the two root compartments also with a significantly higher expression in the half of the root system grown in the untreated ARD soil. The level of gene expression was comparable to the control rhizoboxes with the respective soils in both compartments (Figures 5, 6).

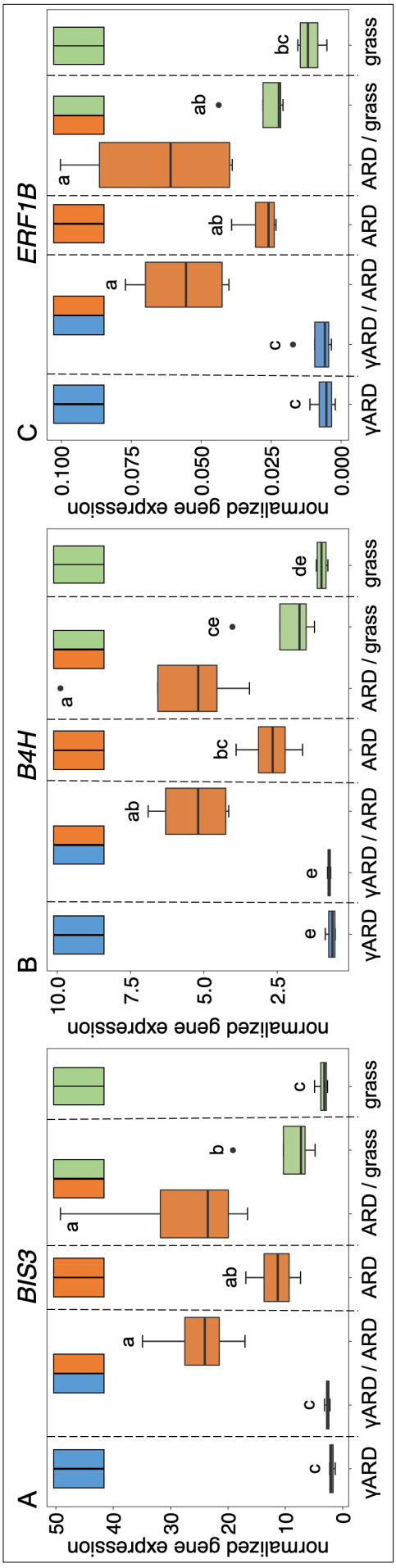

Figure 4: Normalized expression of the ARD candidate genes in experiment 2 analyzed after 2 weeks of culture in split-root chambers filled with γ -disinfected ARD soil (γ ARD), untreated ARD soil (ARD) or grass soil (grass). *Biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase* (*B4H*), *ethylene-responsive transcription factor 1B* (*ERF1B*). Different letters between variants indicate significant differences (Tukey test, $p < 0.05$, $n = 4$).

In the ARD/grass rhizoboxes, the expression of *BIS3* and *B4H* differed significantly between the compartments, with a local elevation in the roots grown in the ARD compartments except for *ERF1B* (Figure 6). However, for *B4H*, the expression in the ARD compartment of the ARD/grass rhizoboxes did not differ significantly from in the ARD compartments of the γARD/ARD rhizoboxes, but was still higher than in the ARD/ARD rhizoboxes (Figure 6B). Overall, ARD marker gene expression was slightly elevated, but not significantly different in the grass/grass from in the γARD/γARD rhizoboxes.

3.4 ARD-associated phenolic compounds and TAC

For the identification of further ARD-related stress responses in the roots, we analyzed the total antioxidant capacity (TAC), total phenolic content (TPC) and the content of single phenolic compounds. In both experiments, the contents of epicatechin and phloridzin were determined. In addition, phloretin was quantified in root extracts of experiment 2.

In experiment 1, the concentrations of all analyzed parameters were elevated in roots grown in compartments with the untreated ARD soil in comparison to those grown in compartments with γ-irradiated ARD soil. The TPC was significantly increased with about 43.9 % in the ARD/ARD chambers compared to the γARD/γARD variants (ARD: 5.9 ± 0.2 mg GAE g^{-1}; γARD: 4.1 ± 0.2 mg GAE g^{-1}, Table 3). In the ARD/γARD rhizoboxes, the TPC was significantly increased in roots of the ARD compartment compared to those in the γARD compartment with 5.3 ± 0.1 and 4.6 ± 0.1 mg GAE g^{-1}, respectively (Table 3). Also, the TAC measured in experiment 1 was significantly higher in roots from the ARD/ARD rhizoboxes (31.5 ± 1.0 mg TE g^{-1} FM) compared to the γARD/γARD variants (23.6 ± 1.0 mg TE g^{-1} FM, Table 3) and likewise differed between the two compartments of the ARD/γARD rhizoboxes. In experiment 2, the same tendency but no statistical differences were found for neither the TPC nor the TAC (Table 3).

The epicatechin content was found to be increased by about 68 % in the ARD/ARD rhizoboxes in comparison to the γARD/γARD rhizoboxes of experiment 1 (3.2 ± 0.3 mg g^{-1} and 1.9 ± 0.1 mg g^{-1} respectively, Table 3). In the ARD/γARD rhizoboxes of experiment 1, also for epicatechin a local increase only in the ARD compartment was recorded. In experiment 2, epicatechin contents showed no statistical differences between the variants (Table 3).

A peak of an unknown compound was detected in the root extracts of experiment 1, which followed the same ARD-specific accumulation pattern as described for the TAC and epicatechin (Table 3). This unidentified compound showed a localized increase in the ARD compartments of the γARD/ARD rhizoboxes with 2.0 ± 0.2 mAU s g^{-1} compared to 1.5 ± 0.1 mAU s g^{-1} in the γARD compartment (Table 3).

The phloridzin contents measured in root extracts of experiment 1 were significantly elevated in the ARD/ARD rhizoboxes compared to the γARD/γARD chambers with 45.9 ± 4.3 and 25.5 ± 1.6 mg g^{-1}_{FM} respectively (Table 3). A significant local increase in phloridzin content was also found in the ARD compartment of the mixed γARD/ARD rhizoboxes with an increase of about 38 % (Table 3). In experiment 2, a similar trend was observed in the extracted phloridzin content (Table 3). Phloretin, the aglycone of phloridzin, was additionally quantified in experiment 2. The trend followed that of phloridzin but only with few significant differences (Table 3).

All measured stress parameters showed a negative correlation with shoot length, shoot fresh mass and root fresh mass (Figure 7). The strongest negative correlation was present for the root fresh mass, ranging from correlation coefficients of -0.56 (TAC) to -0.74 (epicatechin, Figure 7, Tables S1 and S2). Furthermore, the parameters were positively correlated amongst each other with the strongest correlation between epicatechin and phloridzin (correlation coefficient 0.85, Figure 7).

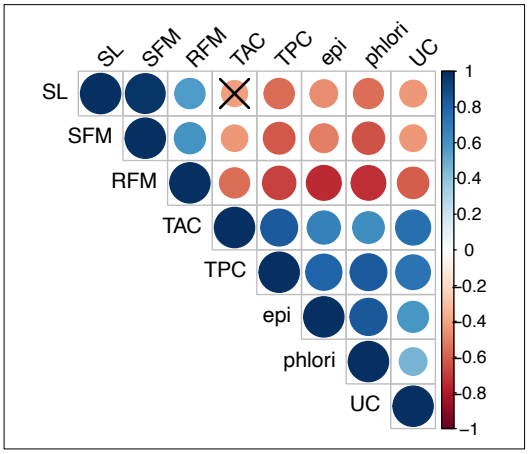

Figure 6: Spearman correlations of growth parameters and experiment 1. Colors depict correlations coefficients and circle size depicts p. Non-significant correlations ($p \geq 0.05$) are crossed out. Shoot length (SL), shoot fresh mass (SFM), root fresh mass (RFM), total antioxidative capacity (TAC), total phenolic content (TPC), epicatechin content (epi), phloridzin content (phlori) and content of unknown compound (UC) after 8 weeks. Correlation coefficients and p-values are depicted in Tables S1 and S2.

Table 3: Means and standard deviations of ARD-associated phenolic compounds, total phenolic content (TPC) and Total antioxidant capacity (TAC). Variants with different letters within one row showed significant differences (Tukey test $p < 0.05$, n = as depicted in Table 1).

Experiment		Substrate combinations in rhizoboxes									
		γARD	γARD	γARD	ARD	ARD	ARD	ARD	Grass	Grass	Grass
1	TAC [mg Trolox Equivalent g^{-1}]	23.61 ± 1.04 b		24.27 ± 1.01 b	27.44 ± 0.82 b	31.51 ± 0.96 a		not analyzed		not analyzed	
	TPC [mg GAE g^{-1} FM]	4.13 ± 0.22 b		4.63 ± 0.11 b	5.32 ± 0.14 a	5.92 ± 0.23 a					
	Epicatechin [mg g^{-1} FM]	1.87 ± 0.065 b		1.90 ± 0.08 b	2.49 ± 0.14 a	3.15 ± 0.28 a					
	Unknown peak [mAU s g^{-1}FM]	1.22 ± 0.176 b		1.47 ± 0.06 b	2.02 ± 0.18 a	2.52 ± 0.21 a					
	Phloridzin [mg g^{-1} FM]	25.50 ± 1.56 bc		25.84 ± 2.04 c	35.63 ± 2.32 ab	45.85 ± 4.34 a					
2	TAC [mg Trolox Equivalent g^{-1} DM]	58.59 ± 4.41 a		62.09 ± 7.70 a	72.03 ± 4.03 a	67.68 ± 3.93 a		78.35 ± 4.92 a	60.27 ± 6.21 a	63.39 ± 5.37 a	
	TPC [mg GAE g^{-1} DM]	6.72 ± 0.76 a		7.07 ± 0.90 a	7.26 ± 0.45 a	8.52 ± 0.78 a		7.70 ± 0.68 a	6.48 ± 0.66 a	6.46 ± 1.10 a	
	Epicatechin [mg g^{-1} DM]	3.27 ± 0.44 a		3.01 ± 0.47 a	2.40 ± 0.13 a	3.04 ± 0.25 a		2.40 ± 0.26 a	2.43 ± 0.29 a	2.36 ± 0.40 a	
	Phloridzin [mg g^{-1} DM]	34.92 ± 4.41 b		36.64 ± 4.11 ab	57.59 ± 4.17 a	50.58 ± 3.94 ab		57.99 ± 6.18 a	39.19 ± 5.87 ab	57.60 ± 3.83 a	
	Phloretin [mg g^{-1} DM]	0.41 ± 0.05 b		0.45 ± 0.06 ab	0.63 ± 0.06 ab	0.58 ± 0.06 ab		0.65 ± 0.02 a	0.44 ± 0.03 ab	0.65 ± 0.08 ab	

4 Discussion

4.1 Plant growth is locally diminished belowground and partially leveled out aboveground

This study is the second investigating the apple plant's reaction towards ARD in a split-root system (Lucas et al., 2018) and the first study to explore molecular reactions of apple roots in this system employing ARD soils from two sites. Our results indicate that the macroscopic reaction towards ARD is strongly localized, which is in agreement with the previous findings of Lucas et al. (2018). In both experiments, we observed typical ARD symptoms in terms of root biomass reduction (Figures 1 and 3) and root browning and discoloration in the compartments filled with ARD soil (Figures 2 and 4). These symptoms have been reported in numerous studies employing different apple genotypes (Yim et al., 2013; Yim et al., 2015; Mahnkopp et al., 2018; Grunewaldt-Stöcker et al., 2019; Reim et al., 2020) and are on a microscopic level characterized by a necrotic cortex and epidermis tissue and a reduction in functional root hairs (Hoestra, 1968; Weiß et al., 2017a; Yim et al., 2015). Furthermore, Lucas et al. (2018) found the roots grown in the compartments filled with ARD soil to be less efficient in the uptake of nitrogen than roots of the same plant grown in γARD soil. Interestingly, despite the severe root damage the plants still had light colored root tips, which continued growing (Figures 2 and 4, Hoestra, 1968; Lucas et al., 2018).

Shoot stunting accompanied by biomass reduction, another typical feature of ARD (reviewed in Winkelmann et al., 2019), was observed in our experiments. When half of the root system was growing in disinfected soil, shoot growth was not reduced proportionally (Figures 1 and 2), which was seen at the end of experiment 1, where shoot fresh mass and length were significantly reduced in the ARD/ARD rhizoboxes in comparison to both the γARD/γARD and γARD/ARD boxes (Figures 1 and S1). In experiment 2, this distribution was not found back, most likely due to the low replicate numbers (Figure 3).

The two soils used in this study were the sandy soil from Heidgraben (Entic Podzol developed from aeolian sand, Mahnkopp et al., 2018) in experiment 1 and the loamy sand from Ellerhoop (Endostagnic Luvisol developed from glacial till, Mahnkopp et al., 2018) in experiment 2, which was also used by Lucas et al. (2018). In the sandy ARD soil from Heidgraben, a stronger local growth reduction was observed than in the Ellerhoop soil in which the same number replantings had been done. Other studies support this observation that ARD effects are stronger in sandy soils, possibly due to lower potential to suppress pathogens (Garbeva et al., 2004; Mahnkopp et al., 2018; Winkelmann et al., 2019; Reim et al., 2020; Rohr et al., 2020).

4.2 ARD-associated marker genes and phenolic compounds show are locally regulated in the roots

After confirming the finding by Lucas et al. (2018) that root growth was locally reduced in ARD-compartments, we further investigated the response in these roots on the molecular level in terms of ARD marker gene expression (Rohr et al., 2020) and on the biochemical level regarding contents of selected phenolic compounds.

We found the expression of all three genes, *BIS3, B4H* and *ERF1B* (Table 2), induced in roots grown in ARD soil. This is in agreement with the studies that lead to their identification as ARD biomarker genes (Weiß et al., 2017a; Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020). In previous studies, we discovered that *BIS3* and *B4H* show a strong and consistent upregulation in roots from ARD soils of different textures and cropping histories in comparison to the same soils disinfected by γ-irradiation (Weiß et al., 2017a; Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020). Furthermore, *BIS3* and *B4H* were upregulated in the roots of different apple genotypes upon confrontation with ARD soil (Reim et al., 2020) and upon infection with *Pythium ultimum*, one of the main causal agents of ARD (Shin et al., 2016; Zhu et al., 2016). In this study, we could observe for the first time that their induction is localized to the roots grown in ARD soil even within the root system of the same plant (Figures 5 and 6).

The expression of *BIS3, B4H* and *ERF1B* in response to ARD does not change significantly in leaf material as has been investigated previously (Weiß and Winkelmann, 2017; Reim et al., 2020), indicating the strong localization of the response to the roots. Since shoot growth is severely affected by ARD, changes in phytohormone levels and signaling are expected to occur in the shoot but until now, these key events in root-shoot communication still have to be identified. Expression of both *BIS3* and *B4H* in the roots however, was strongly negatively correlated with shoot length (Reim et al., 2020). The absence of hormonal changes in the shoot may also be a sign that shoot growth reduction is a result of limited nutrient and water uptake caused by the severe root damage (Yim et al., 2013; Lucas et al., 2018; Grunewaldt-Stöcker et al., 2019).

Aboveground morphology of plants is often in an equilibrium with other morphological features, which is called allometry (Huxley and Teissier, 1936; Lacointe, 2000). Allometric relationships of plant growth are often highly constrained by physical and physiological means which enables the description by mathematical models. The Flow Similarity (FS) model describes how plant growth is dynamically adjusted to optimize water use while at balance with a sufficient structural integrity (Price et al., 2015; Brym and Ernest, 2018). Brym and Ernest (2018) found that allometry models applied even in intensive cultivation systems of apple and cherry where plant growth was influenced by other restrains than in nature (high abundance of water and nutrients but additional physical retrains like pruning or training to influence tree habitus). Likewise, allometry could explain plant growth in our highly artificial split-root system, i.e. shoot growth may be reduced to account for a limited water uptake in roots damaged by ARD, for instance.

Both *BIS3* and *B4H* are coding for key enzymes in the biosynthesis of biphenyls and dibenzofurans, which are phytoalexins in *Malus* and *Pyrus* (Liu et al., 2007; Beerhues and Liu,

2009; Liu et al., 2010; Chizzali and Beerhues, 2012; Khalil et al., 2013; Sircar et al., 2015). These polyphenols act in the plant's response to biotic stressors, mainly fungi (Jeandet et al., 2014). A strong correlation between *BIS3* and *B4H* expression in the roots and phytoalexin content has been shown in previous studies (Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020; Balbín-Suárez et al., under review). The transcription factor *ERF1B* has been described previously to be involved in the biotic stress response (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; Oñate-Sánchez and Singh, 2002). A previous study found that *ERF1B* is also involved in the abiotic stress response in apple roots, since it was induced upon heat stress after the plants had been exposed to 37 °C for three days (Rohr et al., 2020). Lucas et al. (2018) and Balbín-Suárez et al. (under review) showed the local effect of ARD compartments on microbial community compositions in the ARD affected rhizosphere and rhizoplane, to which we see the root reacts with a local response in return.

Although only a very low transcript level was present in ARD soil disinfected by γ-irradiation, these genes were also transcribed when the roots were facing grass soil (Rohr et al., 2020), which we also introduced in experiment 2. Rohr et al. (2020) found comparable levels of gene expression for *BIS3*, *B4H* and *ERF1B* in roots grown in ARD and grass soil for seven days. However, after fourteen days their expression in the ARD soil exceeded that of the grass soil significantly. This led to the hypothesis that *BIS3* and *B4H* are also expressed in response to organisms present in certain healthy soils but at a moderate level, while in ARD soil their expression is boosted. Therefore, their level of expression rather than the mere presence or absence of the transcripts is an indication for ARD (Rohr et al., 2020). In the control rhizoboxes (γARD/γARD and grass/grass) of experiment 2, gene expression was on a similar level in the roots grown in grass and in γARD soil. In the mixed rhizoboxes (ARD/grass), however, an increase in gene expression was observed in grass soil, if the neighboring compartment contained ARD soil (significant for *BIS3*, Figure 6).

Further experiments on the more detailed localization of the molecular ARD response have been conducted (Balbín-Suárez et al., under review). The study of Balbín-Suárez et al. (under review) used a similar setup but with a patch of ARD soil surrounded by healthy soil in a column without physical barriers. In this split-column system, the expression of the candidate genes was investigated within the same root growing through a layer of control soil and ARD soil while adjacent roots grew into healthy soil only. In that system, the authors impressively demonstrated that the plant's molecular reaction was even limited only to the root sections in contact with ARD soil even within one root (Balbín-Suárez et al., under review).

4.3 Local accumulation of ARD-associated phenolic compounds

Antioxidant secondary compounds like carotenoids, flavonoids and tocopherols belong to the plant's non-enzymatic antioxidant system. They protect the plants from high concentrations of reactive oxygen species (ROS), which are often triggered by biotic and abiotic stress factors and can dramatically damage the plant cell tissues and can cause a loss of cell function or even cell death (Apel and Hirt, 2004; Kulbat, 2016; Choudhary et al., 2020).

In experiment 1, results indicate that the roots' response in ARD soil was the accumulation of antioxidant and phenolic compounds, especially epicatechin, phloridzin and another unknown compound. The browning observed in roots affected by ARD is believed to be caused by the oxidation of these compounds (Yim et al., 2013). In the mixed γARD/ARD boxes these phenolic compounds were increased in the roots grown in the ARD compartment but not in those of the γARD compartment (Table 3). This indicates a local stress response in apple roots faced with ARD soil, which we observed here for the first time and which supports the reports on local changes in microbes and nitrogen uptake (Lucas et al., 2018) as well as ARD marker gene expression (this study).

The increase of phloridzin in apple roots in response to ARD soil has been recorded by Emmett et al. (2014). It was furthermore shown by Emmett et al. (2014) that the relative spectrum of phenolic compounds depended on the root's developmental stage: Pioneer and first order roots had higher relative amounts of several phenolic compounds, while the relative phloridzin content was higher in third order roots. Therefore, the authors assumed that phloridzin may be necessary to protect the vascular tissue in third order roots from pathogens (Emmett et al., 2014). Furthermore, in a recent study of rose root secondary metabolites in the context of replant disease, phloridzin was suggested to be a phytoanticipin rather than a phytoalexin (Yim et al., 2020). Future studies should consider a sampling of different root orders and include analyses of root exudates.

We also detected the monomer epicatechin, a flavan-3-ol, with higher concentrations in roots grown in ARD soil of experiment 1 (Table 3). Epicatechin has antioxidative properties and inhibits lipoxygenase activity. This was especially shown in the pericarp of avocado fruits until ripening (Prusky et al., 1985). Epicatechin is also involved in the antifungal response against *Venturia inaequalis* in apple fruits and leaves (Mayr et al., 1997; Michalek et al., 1998). In rose roots, epicatechin contents were highly variable in response to rose replant disease (Yim et al., 2020), which is similar to the high variability of epicatechin we detected in apple roots (Table 3). Furthermore, it has been proven that root extracts of *Aruncus silvester* and *Potentilla alba*, both members of the Rosaceae family, are rich in procyanidins, the precursor of which is the monomer (-)-epicatechin (Oszmianski et al., 2007). Both procyanidin B2 and (-)-epicatechin are responsible for oxidative browning of litchi pericarp (Liu et al., 2010). For this reason, we assume that epicatechin amongst other phenolic compounds is involved in discoloration of *Malus* roots.

Phenolic compounds, such as epicatechin and catechin have high antimicrobial activity, which was shown for several esterified phenolic compounds, e.g. gallats (Friedman et al., 2006). The

esterification increases water solubility and stability of these compounds), which confers an antimicrobial activity similar to medical antibiotics (Krygier et al., 1982). Muthuswamy and Rupasinghe (2007) assessed the antimicrobial activity of catechin, chlorogenic acid and phloridzin against *E. coli*, *Listeria innocua*, and *Penicillium chrysogenum* and found their growth suppressed by all the phenolics. In addition, phenolic acids (e.g. chlorogenic acid), flavan-3-ols (e.g. epicatechin), flavonols (quercitrin), and dihydrochalcones (phloridzin and phloretin) were locally increased in scab infected apple leaf tissue and fruit peel compared to healthy tissue (Petkovšek et al., 2009) supporting their role in disease response.

The unknown compound detected at 21.3 min had its absorption maximum at 254.4 nm followed by 280.4 nm, but no signal could be detected at 320.4 nm and 370.4 nm. In experiment 1, no further wavelengths had been tested, and the methodical changes between experiments 1 and 2 caused difficulties to identify this compound.

Several factors might be responsible for the fact that the phenolic compounds detected in experiment 2 did not exactly show the same patterns as observed in experiment 1. One explanation is the use of different soil origins as discussed above. Furthermore, extraction was done using fresh root material in experiment 1, while 100 mg freeze-dried root material was used for experiment 2. Lastly, experiment 2 included comparably low replicate numbers due to the limitation of available rhizoboxes, which in combination with the high variability of the phenolic compounds limited the statistical power.

4.4 The usefulness of a local biotic stress response and possible implications on ARD countermeasures and biotests

Plant defenses are considered an energetic trade-off between protection and vegetative or generative growth (discussed in Zhu and Saltzgiver, 2020). A targeted defense response addressing only the affected tissue has the advantage of focusing the energy spent of affected

areas but possibly at a cost of reaction speed. Biphenyl and dibenzofuran phytoalexins play a major role in the localized biotic stress response of apple towards ARD in the roots as we saw in this study by the local induction of the phytoalexin biosynthesis genes *BIS3* and *B4H*. It has been shown furthermore, that *BIS3* is induced in apple shoots upon *Erwinia amylovora* infection accompanied by increased concentrations of biphenyl and dibenzofuran phytoalexins (Chizzali et al., 2012a; Chizzali et al., 2012b; Chizzali et al., 2013). Weiß et al. (2017b) hypothesized that high local concentrations of phytoalexins produced by apple in order to combat ARD could have detrimental effects on the root tissue itself, since an autotoxic effect of various phytoalexins has been shown for other systems (e.g. Glazener, 1978; Rogers et al., 1996). In the case of autotoxicity caused by the defensive compounds, a highly localized response would also be beneficial for the plant to reduce the damage to itself.

On the other hand, Zhu et al. (2017) reported indicators of a preformed defense in the *Pythium* resistant apple genotype G935 in comparison to the susceptible genotype B9, which may stand against the growth versus trade-off theory. However, preformed protection mechanisms allow for a much faster biotic stress response. Zhu et al. (2018) demonstrated the importance of a rapid response in 'Ottawa 3' x 'robusta 5' crossings in response to *Pythium ultimum* attack. Susceptible genotypes showed quickly-spreading root necrosis already 24 h after post inoculation, which was delayed by several days by the formation of a physical barrier hindering hyphae growth in the resistant genotypes.

Only a direct or very close contact with the ARD microbiome and possible involved volatiles has an impact on the root morphology (Lucas et al., 2018; Balbín-Suárez et al., under review; this study). This is in agreement with the observations of Hoestra (1968) in the field, where young replanted trees suffered the most from stunted growth but became more tolerant once they explored deeper areas of the soil less affected by ARD. Also in our experiments we observed a difference in shoot growth when not all of the root system was exposed to ARD soil.

Dilution of the ARD soil with 20 – 95 % healthy soil without spatial separation did not yield these strong effects on ARD symptom reduction (Hoestra, 1968; Jaffee et al., 1982; van Schoor et al., 2009; Tewoldemedhin et al., 2011b; Spath et al., 2015). This dilution effect has to be considered when assessing and counteracting ARD in the field. Inter-row planting is a common strategy that is facilitated by both the low mobility of ARD in the soil and possibly the dilution effect once the plants start exploring the soil around them.

5 Conclusions

Our split-root approach confirmed the local root growth response of apple roots towards ARD using ARD soil of two sites differing in soil texture and organic matter content. The ARD marker gene expression and the spectrum of phenolic compounds analyzed also showed a clear local response. Future studies should encounter the patchy occurrence of ARD causal agents and should focus on analyzing root segments according to their order, ontogenetic development (root tips, elongation zone, and older root segments) as well as the ARD severity observed on the respective segment. Additionally, a focus will be on the identification of unknown phenolic compounds involved in the ARD stress response by GC-MS, LC-MS/MS and HPLC-HRMS. The fact that the plants are leveling out shoot growth has to be considered in evaluating ARD severity in the field and in the development of new countermeasures.

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5 Root exposure to apple replant disease soil triggers local defense response and rhizoplane microbiota dysbiosis

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Abstract

A soil column split-root experiment was designed to investigate the ability of apple replant disease (ARD) causing agents to spread in soil. 'M26' apple rootstocks grew into a top layer of Control soil, followed by a barrier-free split-soil layer (Control soil/ARD soil). We observed a severely reduced root growth, concomitant with enhanced gene expression of phytoalexin biosynthetic genes and phytoalexin content in roots from ARD soil, indicating a pronounced local plant defense response. Amplicon sequencing (bacteria, archaea, fungi) revealed local shifts in diversity and composition of microorganisms in the rhizoplane of roots from ARD soil. An enrichment of OTUs affiliated to potential ARD fungal pathogens (*Ilyonectria* and *Nectria* sp.) and bacteria frequently associated with ARD (*Streptomyces*, *Variovorax*) was noted. In conclusion, our integrated study supports the idea of ARD being local and not spreading into surrounding soil, as only the roots in ARD soil were affected in terms of growth, phytoalexin biosynthetic gene expression, phytoalexin production, and altered microbiome structure. This study further reinforces the microbiological nature of ARD, being likely triggered by a disturbed soil microbiome enriched with low mobility ARD-causing agents that induce a strong plant defense and rhizoplane microbiome dysbiosis, concurring with root damage.

Keywords: split-root experiment, phytoalexins, soil microbiome, root system architecture, X-ray computed tomography.

1 Introduction

Apple replant disease (ARD) is a phenomenon that causes reduced plant and fruit quality and yield and, as a consequence, important economic losses to apple (*Malus* sp.) nurseries and orchards worldwide. Upon replanting of new apple trees at the same site where the previous generations were grown, a strong depression in plant growth can be observed (Mazzola and Manici 2012; Winkelmann *et al.* 2019). ARD aboveground symptoms include general stunting, shortened internodes and deformed leaves, whereas belowground root growth arrestment and necrosis occur (Savory 1966; Hoestra 1968; Grunewaldt-Stöcker *et al*. 2019). These symptoms are most likely a consequence of a strong, but impaired, plant defense or stress response to ARD soil that eventually could lead to autotoxicity (Hofmann *et al.* 2009; Henfrey, Baab and Schmitz 2015; Weiß *et al*. 2017). Especially plant defense molecules like phytoalexins, which are usually produced by Malinae species against fungal and bacterial pathogens, were found in strikingly high concentration in ARD-affected roots of several *Malus* genotypes (Weiß *et al*. 2017; Reim *et al*. 2020). Likewise, genes involved in phytoalexin biosynthesis are specifically up-regulated in roots of plants exposed to ARD and have been, therefore, suggested as early genetic biomarkers for ARD (Rohr *et al.* 2020). These findings, coupled with the fact that disinfection treatments overcome the disease, point toward microorganisms as plausible causing agents (Yim, Smalla and Winkelmann 2013; Balbín-Suárez *et al*. 2020). Although many efforts have been made to elucidate ARD etiology, its causality is still actively debated (Winkelmann *et al*. 2019). Evidence is progressively accumulating toward the involvement of a "microbial community property" rather than one or several causing agents, often referred to as a "soil microbiome dysbiosis" (Lucas *et al*. 2018; Nicola *et al*. 2018; Balbín-Suárez *et al*. 2020). However, although the root architecture, the plant defense response, and the microbiome analysis were previously investigated in separate studies, no multiphasic approach including these factors was attempted before.

ARD is also defined by other specific and less studied traits, i.e. specificity, lack of spreading into surrounding soils, reversibility and persistence (Savory 1966; Hoestra 1994), the better comprehension of which might be crucial for elucidating and delimiting ARD causality. One of the most intriguing ARD-specific characteristics is the apparent inability of the causing agents to spread into the surrounding soil in the field, unlike other plant disease-causing pathogens, e.g. wilt or dieback diseases caused by *Ralstonia solanacearum*, *Fusarium oxysporum f.* sp. *cubense (Foc)* and *Phytophthora cinnamomi* (Ristaino and Gumpertz 2000;Dita *et al*. 2019; Elsayed *et al*. 2020). Indeed, ARD seems to be confined locally to the very same planting hole where previous trees were grown. Back in 1968, Hoestra noted this inability to spread, as apple plants could recover once roots reached deeper non-affected layers after passing through an ARD soil, a phenomenon also observed for other plant species affected by replant disease, e.g. marram grass (Van der Putten 1989; Hoestra 1994). Moreover, some orchard management practices reinforce the "low mobility of ARD-causing agents" aspect, as planting new trees slightly away from the previous implantation spots (e.g. at the interspace, at the traffic lanes) or directly into the implantation spots but after soil renewal (e.g. fresh soil), seemed to alleviate ARD severity (Savory 1966; Hoestra 1968; Mai and Abawi 1981; Kelderer *et al*. 2012).

Recent experiments showed in a split-root approach, with a physical barrier between ARD-affected and Control soil, that ARD is not systemic within the plant (Lucas *et al.* 2018). Indeed, exposure of apple roots to ARD soil resulted in the selection and enrichment of very specific microbiome in the microhabitats directly under plant influence, i.e. the rhizosphere and the rhizoplane, concomitant with local plant symptoms (Lucas *et al.* 2018; Balbín-Suárez *et al*. 2020). However, this former split-root approach based on a physical barrier between compartments could not determine whether the disease-causing agents are indeed able to spread from an ARD patch to the surrounding soil. Additionally, in previous experiments we could

observe that 'M26' plantlets avoided growing into columns only filled with ARD soil, forming a quasi-horizontal root system within the gravel layer covering the columns (unpublished), suggesting the existence of plant negative (chemo)tropisms towards something present in ARD soils, e.g. toxins, volatiles (Wenke *et al.* 2012; Lee *et al.* 2016).

To shed light on the ARD low mobility aspect, we developed a novel split-root design in soil columns assembled from different substrates in concomitant layers without any physical barrier, allowing the natural flow of microorganisms and molecules, e.g. toxins, volatiles (Fig. 1). Roots of the ARD sensitive 'M26' rootstock were progressively growing through different substrates, including a first layer of Control soil to initiate healthy root growth, followed by a split-layer containing ARD soil on one side, and Control soil on the other side. Since ARD-causing agents are unknown, it means a challenge to study their mobility or spread abilities in soil. Thus, the present study aiming at providing insights into the low mobility of ARD-causing agents was based on the study by Lucas *et al.* (2018) demonstrating that apple root reactions towards ARD soil were local and not systemic. If ARD-causing agents do not spread through the soil, only roots exposed to the ARD soil patch will be affected by disease, whilst roots exposed to the Control soil patch will remain healthy. To achieve this goal, we set an integrated approach by investigating the response of plant roots to ARD soil at: i) the gene expression level using ARD transcriptional biomarkers (phytoalexin biosynthetic genes; RT-qPCR), ii) the molecular level by measuring plant defense compounds (phytoalexin content; GC-MS), iii) the microbial level by amplicon sequencing of the soil and the rhizoplane microbiome (bacteria, archaea, fungi; Illumina MiSeq) and iv) the root architecture level by X-ray computed tomography (CT) and WinRHIZO scans (root development and microorganisms travel distances). Additionally, the specific substrate layer disposition allowed us to investigate a further research question, i.e., whether roots of the ARD-sensitive rootstock 'M26' sense, and avoid growing into ARD soil, as shown in the aforementioned pre-experiments. Finally, since

concerns were previously raised on the potential deleterious effects of CT scanning on root growth and microbial community structures (Fischer *et al*. 2013; Blaser *et al*. 2018), we have also set two CT scan frequency treatments (low and high frequencies) to evaluate whether our detection method is generating deleterious effects that might hamper the conclusions drawn from this study.

2 Material and Methods

2.1 Barrier-free split-root experiment under composite soil column design

A split-root experiment was set up in columns (n = 20) assembled from different substrates (Control, ARD) in concomitant layers without any physical barrier (L1 to L4, Fig. 1). This was arranged to lure root growth through an ARD-affected soil patch, while also giving the plant the chance to bypass it (split-layer). The soil (endostagnic Luvisol with 73 % sand, 15 % silt and 10 % clay, from Ellerhoop, Chamber of Agriculture Schleswig-Holstein, Germany) originated from a reference site of the ORDIAmur consortium (www.ordiamur.de) and was previously used in ARD studies (Mahnkopp *et al.* 2018; Lucas *et al.* 2018; Radl *et al*. 2019; Rohr *et al.* 2020; Balbín-Suárez *et al*. 2020). The full soil description is available in Mahnkopp *et al.* (2018). ARD was induced at the Ellerhoop site by replanting apple seedlings ('Bittenfelder Sämling') every two years since 2009. Grassland soil from adjacent plots served as control (Control). The soil in the split-layer (ARD-L2/Control-L2) was sieved (1 mm mesh) to maximize contrasts *via* X-ray computed tomography (CT) scans. Upper and lower layers (L1, L3 and L4) contained 2 mm sieved Control soil (grassland soil). Soil was filled into plastic cylinders (25 cm height, 7 cm diameter, 1.1 g cm^{-3} bulk density). A top 1 cm gravel layer was added to limit evaporation. Inert garnet grains were used to distinguish both sides. Nutrients were supplied with 2 g kg^{-1} Osmocote exact 3-4 M (16-9-12+2MgO+trace elements; https://icl-sf.com).

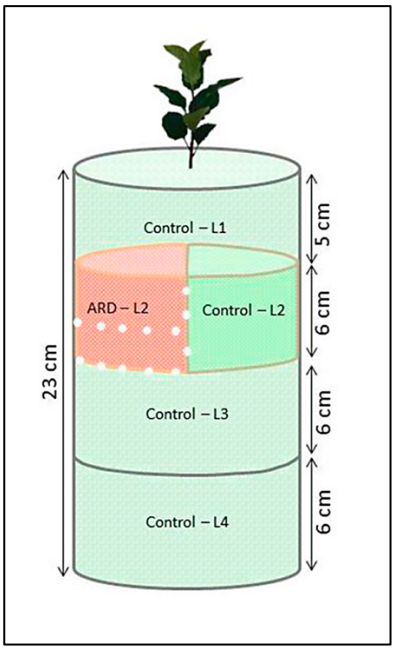

Figure 1. **Sketch of a split root column.** In 5-11 cm depth half of the layer was filled with ARD soil (marked in red).

2.2 Plant material and growth conditions

In vitro propagated and acclimatized apple plants (rootstock genotype 'M26') were transplanted in columns wrapped with aluminum foil to prevent algal growth. Columns were placed in a climate chamber (29 days, 20-18 °C day-night, 70 % relative humidity, 16 h photoperiod at 350 µmol m^{-2} s^{-1}) on KERN 572 weighing cells (Kern & Sohn GmbH, Balingen, Germany) for controlled irrigation. Plants were watered initially to 18 % volumetric water content, and kept constant by watering every second day. Two thirds of the water was applied from the top, one third from the bottom, allowing constant water movements to exclude a lack of dispersion of ARD due to water-deficiency. Each experimental unit received one plantlet (n = 20).

2.3 X-ray computed tomography

Two CT-scan modalities were tested: A weekly-scanning modality (frequent CT: “fCT”, n = 10) and an end-point scanning modality (single CT: “sCT”, n = 10). In the fCT treatment, columns were scanned after 14, 21 and 28 days to follow root development, while in the sCT treatment columns were scanned once after 28 days, one day before harvest. X-ray tomography was performed with an industrial µCT (X-TEK XTH 225, Nikon Metrology, Alzenau,

Germany) located next door to the climate chamber. CT-settings for large images (whole column diameter) were 140 kV, 286 µA and 500 ms exposure time. A total of 2,748 one-framed projections were applied (total exposure time: 23 min). Copper filters were used (0.5 mm, X-ray source/sample distance: 23.7 cm, resolution: 45 µm, dose: 0.49 Gy [sCT] and 1.46 Gy [fCT]). Improved resolution was achieved on subsamples collected at harvest (135 kV, 150 µA, 2,500 projections/one frame per projection, total exposure time: 29.5 min, dose: 1.8 Gy, resolution: 19 µm). Analysis of subsampled CT scans was done with the adapted "*Rootine*" algorithm (Gao *et al*. 2019).

2.4 Sampling of roots, soil and subsampling

Aboveground parts of apple plants were cut with sterile scissors. One half of the replicates (n = 5 columns) was used for microbiome sampling and the other half for gene expression and phytoalexin sampling (sample overview; Table S1, Supporting Information). For both types of analysis, samples were extracted layer-wise. For the microbiome sampling, roots were vigorously shaken and attached soil was brushed off and discarded. At this step, roots were used to recover the rhizoplane by vigorous washing in 50 mL Falcon tubes (30 mL distilled sterile water, 1 min, hand shake), followed by centrifugation (10,000 *g*, 30 min, 4 °C). Pellets were recovered and frozen (-80 °C) until DNA extraction. For gene expression and phytoalexin content analyses parts of roots were cleaned from adhering soil and snap-frozen (liquid nitrogen, -80 °C storage).

In addition to the sampling procedure described above, for all replicates undisturbed subsamples were taken from both compartments of the split-layer (Fig. 1) by pressing an aluminum cylinder (height: 3 cm, Ø = 3 cm) into the center of each soil substrate patch. The split-layer was pushed-out and the subsamples were carefully removed from the rest of the compartments. After subsample CT scans (see above), the roots were recovered by washing and used for WinRHIZO analysis (see next section). The roots remaining were preserved in

Rotisol and stored at 4 °C until analysis for length and diameter class. For roots used for gene expression analysis a picture was taken including a scale bar – the length of these roots was also included in the WinRHIZO analysis.

2.5 Measurement of root length and diameter classes

Total length and diameter classes were measured from roots taken by destructive sampling at harvest, and from subsamples after CT scanning (10 replicates), using the WinRHIZO software (2009b, Regent Instruments, Canada; https://regent.qc.ca/index.html). Root length was determined in 11 root diameter classes ranging from < 0.05 mm to > 2 mm. The initial diameter classes were merged into four groups (≤ 0.25 mm, ≤ 0.50 mm, ≤ 0.75 mm and ≥ 1.00 mm).

2.6 Gene expression analysis

Frozen root samples (100 mg, 4 mm steal beads) were homogenized, RNA was isolated as previously described (Rohr *et al.* 2020) and stored at -80 °C until cDNA synthesis. First strand cDNA synthesis was carried out with the RevertAid First Strand cDNA Synthesis Kit (1 µg RNA input, random hexamer primers; Thermo Scientific, Waltham, MA, USA) and 1:5 diluted cDNA was stored at -20 °C until qPCR measurements.

Reactions were run on a CFX Connect instrument (Bio-Rad, Hercules, CA, USA) using the SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Primers for the three ARD-early biomarker genes (Table S2, Supporting Information) *biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase* (*B4H*) and *ethylene-responsive transcription factor 1B-like* (*ERF1B*) were selected as previously described (Rohr *et al.* 2020). *Elongation factor 1-α* (*EF1a*), *elongation factor 1-β-* (*EF1b*) and *tubulin beta chain* (*TUBB*) (Weiß, Bartsch and Winkelmann 2017) were chosen as reference genes for normalization after testing their expression stability. qPCR was performed as previously described (Rohr *et al.* 2020) with two technical replicates for *BIS3, B4H, EF1a, EF1b* and *TUBB* (three for the *ERF1B* gene with low expression). Normalized gene expression was calculated according to Pfaffl (2001).

The statistical evaluation was performed using R version 3.5.1 (R Development Core Team 2011) in RStudio version 1.1.456. First, the data were checked for a Gaussian distribution and log_{10}-transformed, if necessary. A linear model was fitted and an analysis of variance (ANOVA) was performed. Tukey's all-pairwise comparisons of means were performed with the *multcomp* (package version 1.4-8; Hothorn *et al*. 2008).

2.7 Phytoalexin extraction and analysis

Aliquots of root samples from gene expression analysis (Table S1, Supporting Information) were lyophilized and homogenized to fine powder (29 Hz, 1 min; Mixer Mill MM400, Retsch, Haan, Germany). Extraction and analysis of phytoalexins were conducted as previously described (Weiß *et al.* 2017). Methanol (1 mL) containing 50 µg of 4-hydroxybiphenyl (internal standard for relative quantification) was added to the samples, which were continuously vortexed (2,700 rpm, 20 min; Vortex Genie 2, Scientific Industries, Bohemia, NY, USA). The resulting extracts were centrifuged (13,439 *g*, 10 min) and aliquots (200 µL) of the supernatants were air-stream dried in reagent tubes. Residues were dissolved in 200 µL ethyl acetate, centrifuged (13,439 *g*, 10 min) and supernatants were transferred to gas chromatography-mass spectrometry (GC-MS) vials with glass inlet. Ethyl acetate was air-stream evaporated and the residues were re-dissolved in 50 µl N-methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA, ABCR, Karlsruhe, Germany) and incubated at 60 °C during 30 min for silylation. Silylated samples were analyzed by GC-MS (70°C for 3 min, 70°C-310°C in 24 min [10°C/min] and 310°C for 5 min, helium flow: 1 mL min^{-1}, injection volume: 1 µL, split ratio 1:10).

2.8 Total community DNA extraction

Extraction of total community DNA was done from entire rhizoplane pellets and from 0.5 g of bulk soil (FastDNA® SPIN Kit for soil and FastPrep® Instrument, MP Biomedicals, Santa Ana, CA, USA) followed by DNA purification (GENECLEAN® SPIN Kit, Qbiogene Inc., Carlsbad, CA, USA) as previously described (Lucas *et al*. 2018).

2.9 Library preparation

To study the bacterial/archaeal and fungal communities, the hypervariable V3-V4 region of the 16S rRNA gene and the fungal ITS2 region were amplified by PCR and sequenced (n = 5; Miseq® Illumina®, San Diego, CA, USA) according to acknowledged practice guidelines (Schöler *et al*. 2017).

For 16S rRNA gene amplification, primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used (Sundberg *et al.* 2013; Caporaso *et al.* 2011). Reactions were performed as previously described by Babin *et al.* (2019), except that NEB HotStart Taq and NEB Standard reaction buffer were used (New England Biolabs, Ipswich, MA, USA).

For ITS2 fungal region amplification, primers gITS7 (5'-GTGARTCATCGARTCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ihrmark *et al*. 2012) were used in a 25 µL PCR reaction volume containing: 1 µL TC-DNA, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 5% (v/v) dimethyl sulfoxide (DMSO), 1X NEB Standard Reaction buffer and 0.625 U of NEB HotStart Taq (New England Biolabs, Ipswich, MA, USA). PCRs were done as follows: initiation (95°C, 5 min), 30 cycles of denaturation (95°C, 30 s), annealing (56°C, 30 s) and extension (72°C, 1 min), with final extension (72°C, 10 min).

A second PCR step was done to include Illumina sequencing adapters and unique dual-index combinations for each sample. After both PCRs, amplicons were purified to remove short fragments < 100 bp (0.65:1 beads:PCR volume ratio; HighPrep™ PCR Clean Up System, AC-60500, MagBio Genomics Inc., MD, USA), normalized (SequalPrep Normalization Plate 96 Kit, Invitrogen, Maryland, MD, USA) and pooled. The pooled library was concentrated (DNA Clean and Concentrator™-5 kit, Zymo Research, Irvine, CA, USA), quantified (Quant-iT™ High-Sensitivity DNA Assay Kit, Life Technologies; *www.lifetechnologies.com*) and adjusted to 4 nM before denaturation and loading. Amplicon sequencing was performed on an Illumina

MiSeq platform using Reagent Kit v2 [2 x 250 cycles] (Illumina Inc., San Diego, CA, USA). Unassembled raw amplicon data is available at the Sequence Read Archive public repository (SRA; *https://www.ncbi.nlm.nih.gov/sra*) under the accession number PRJNA644274 (*http://www.ncbi.nlm.nih.gov/bioproject/644274*).

2.10 16S rRNA gene and ITS amplicon sequences processing

Primer sequences were removed (cutadapt; Martin 2011). Only read pairs with successful primer removal were considered for further analysis. Primer-trimmed 16S rRNA gene sequences were merged (*assemble_pairs*) and clustered in Operational Taxonomic Units (OTUs, 97%, *cluster_otus*) with UPARSE-OTU algorithm (Edgar 2013) using a custom BioDSL script (*https://github.com/maasha/BioDSL*). Primer-trimmed ITS2 sequences were processed (ITS-dedicated PIPITS workflow, version 2.2; Gweon *et al*. 2015), merged (PEAR; Zhang *et al*. 2014) and quality-filtered (FASTX-Toolkit, *http://hannonlab.cshl.edu/fastx_toolkit/index.html; Key: citeulike: 9103573*). ITS2 subregions of fungal origin were extracted from sequences (ITSx; Bengtsson-Palme *et al*. 2013) and clustered into OTUs (VSEARCH, 97%). Taxonomic annotations of 16S rRNA gene OTUs cluster representatives were performed with mothur (Schloss *et al*. 2009) naïve Bayes' classifier using Ribosomal Database Project database trainset 16 (Cole *et al*. 2014) formatted for mothur (*https://www.mothur.org/wiki/RDP_reference_files*). Representative ITS OTU sequences were screened for chimeras (UNITE-UCHIME reference dataset), and assigned taxonomy (RDP Classifier, UNITE fungal ITS reference database, version 7.2; *https://doi.org/10.15156/BIO/587478*).

A phylogenetic tree was constructed with representative 16S rRNA gene OTU sequences (mafft version 7.407 with retree 1; Katoh and Standley 2013), and an approximate ML tree was made and rooted at midpoint (FastTree version 2.1.10; Price, Dehal and Arkin 2010). Sequencing completion was estimated with Good´s coverage index (Good 1953) and rarefaction curves

(*vegan* package R) (Table S3 and Fig. S1, Supporting Information). Samples with less than 2,000 sequences and/or low coverage (Good's coverage < 97 %) were excluded from further analysis (Caporaso *et al*. 2011). Subsequent data analysis was conducted using packages developed for the R software version 3.4.1 (R Core Team 2017).

2.11 Sequencing data analysis

For alpha-diversity analysis, 16S rRNA gene and ITS2 samples were rarefied to 8,000 counts to avoid biases due to uneven sequencing depth (samples < 8,000 counts not included; Table S3, Supporting Information). The following indices were considered: Richness, ACE (Abundance Coverage Estimator), Shannon, Simpson and Simpson reciprocal (*vegan* R package; Oksanen *et al*. 2019). Statistical differences between treatments were assessed by generalized linear model (GLM) and a post-hoc Tukey´s HSD correction test ($P < 0.05$, *multcomp* R package; Hothorn *et al*. 2008) and Kruskal-Wallis test ($P < 0.05$, *agricolae* R package; Mendiburu 2015) for normal and not normal distributed data, respectively. Normal data distribution was assessed by D´Agostino test of skewness (Table S4, $P < 0.05$, *moments* R package; Komsta and Novomestky 2015).

To study the effect of investigated factors (microhabitat: bulk soil *vs*. rhizoplane; soil substrate: Control *vs*. ARD; and CT frequency: single *vs.* frequent) on the microbial community composition at the OTU level, a Constrained *Analysis* of Principal Coordinates (*CAP*), i.e. a distanced-based redundancy analysis, was applied on relative abundance normalized data (Bray-Curtis dissimilarity index, capscale function, 10,000 permutations, *vegan* R package). The significance of root length on the beta-diversity index used (here Bray-Curtis) was calculated by PERMANOVA (adonis, *vegan* R package; Oksanen *et al*. 2019). Taxa that contributed to changes in the community composition were identified at phylum level (relative abundance, ANOVA under GLM with Tukey'HSD posthoc test, $P < 0.05$, *multcomp* R package; Hothorn *et al.* 2008) and OTU level (raw counts, quasi-likelihood F-test under

negative binomial generalized linear modeling [nbGLM] with false discovery rate adjustment, FDR-adjusted $P < 0.05$, *edgeR* package; Robinson, McCarthy and Smyth 2010). At the OTU level, the identified taxa that were significantly enriched or depleted in ARD-L2 in comparison to Control-L2 samples were designated as "responders". Since differences attributed to CT scanning frequency were marginal for both bacterial/archaeal and fungal datasets, significant differences in OTU abundance between Control-L2 and ARD-L2 substrates were calculated by aggregating sCT and fCT profiles.

3 Results

3.1 Substrate and depth-dependent root growth

Roots did not completely bypass the ARD-L2 layer (Fig. 2A). They initially grew into ARD-L2 as into Control-L2 (14 days after planting); growth within ARD-L2 significantly slowed down hereafter 21 to 28 days after planting, hence featuring less roots with only short laterals compared to Control-L2. Since 83 % of the root length had a diameter < 0.5 mm and 36 % even less than < 0.25 mm (Fig. S2, Supporting Information), automatic root segmentation was impossible in overview CT-scans. Therefore, all further reported CT data refer to the subsamples taken from the split-layer. No significant differences were observed in shoot biomass and root length between the two CT-scan frequencies. Roots in Control-L1 were well developed, showing overall the highest root length densities. In comparison, ARD-L2 had significantly lower root length densities in both sCT and fCT (Fig. S3, Supporting Information). This was also observed by CT-scanning of split-layer subsamples (Fig. 2B). Root length estimation from either CT scans or WinRHIZO on subsamples showed a strong positive correlation ($R^2 = 0.79$). A decline in fine roots was observed in ARD soil (27 % < 0.25 mm) compared to Control soil (36 % < 0.25 mm; Fig. S2, Supporting Information). The marked

differences in root growth between ARD-L2 and Control-L2 resulted in a higher frequency of long distances for diffusion/mobility from any point in the soil to the root surface (Fig. 2C).

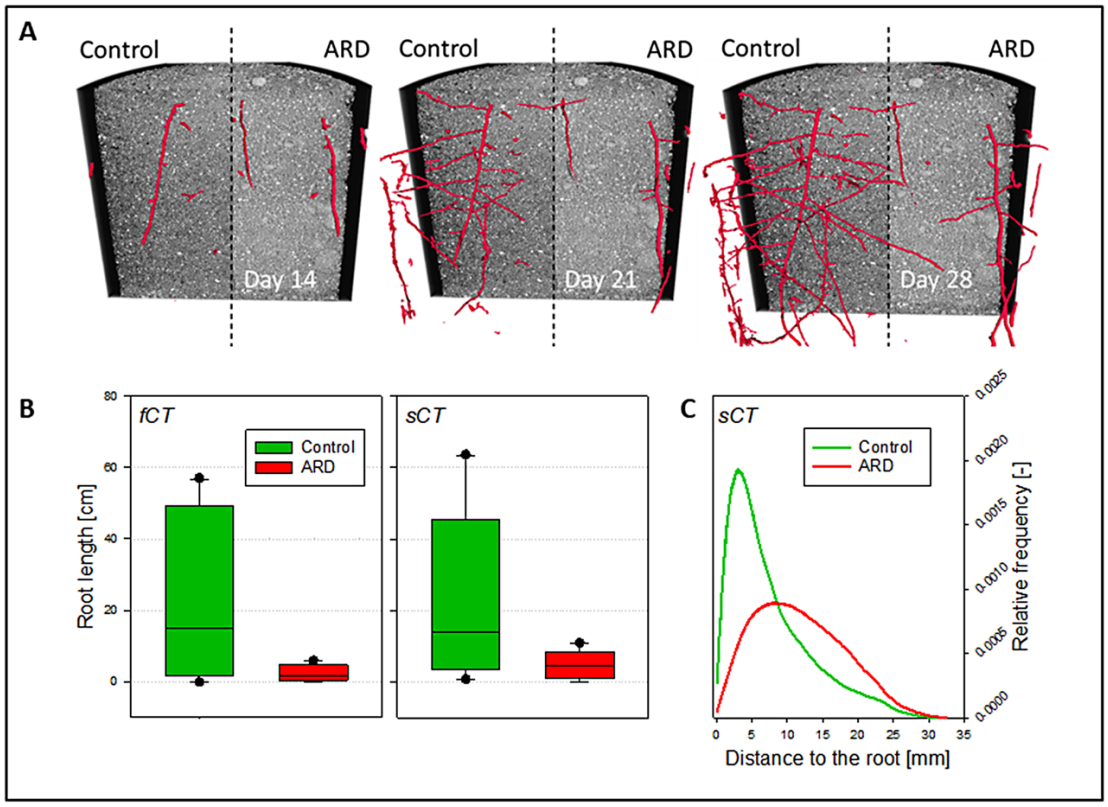

Figure 2. (A) Development of the root system over time (day 14, 21, 28 after planting) in the split layer. 3D visualization of segmented roots in X-ray CT scans; roots are shown in red, soil matrix in grey scale, column walls are visible in black. (B) Box-plot representation of root length based on segmented root systems from CT images of subsamples from Control-L2 and ARD-L2, respectively (n=10) 29 days after planting. (C) Frequency distribution of potential travel distances from any point in the investigated soil volume to the nearest root surface, derived from euclidean distance transformation conducted from X-ray CT images after root segmentation. For details of the concept see (Schlüter et al. 2018). fCT = frequently scanned treatment, sCT = single X-ray CT scan prior to harvest.

3.2 Expression of ARD-biomarker genes in roots

Expression levels of *BIS3*, *B4H* and *ERF1B* did not differ significantly between CT frequencies (see values, Fig. S4, Supporting Information), thus data were combined (Fig. 3).

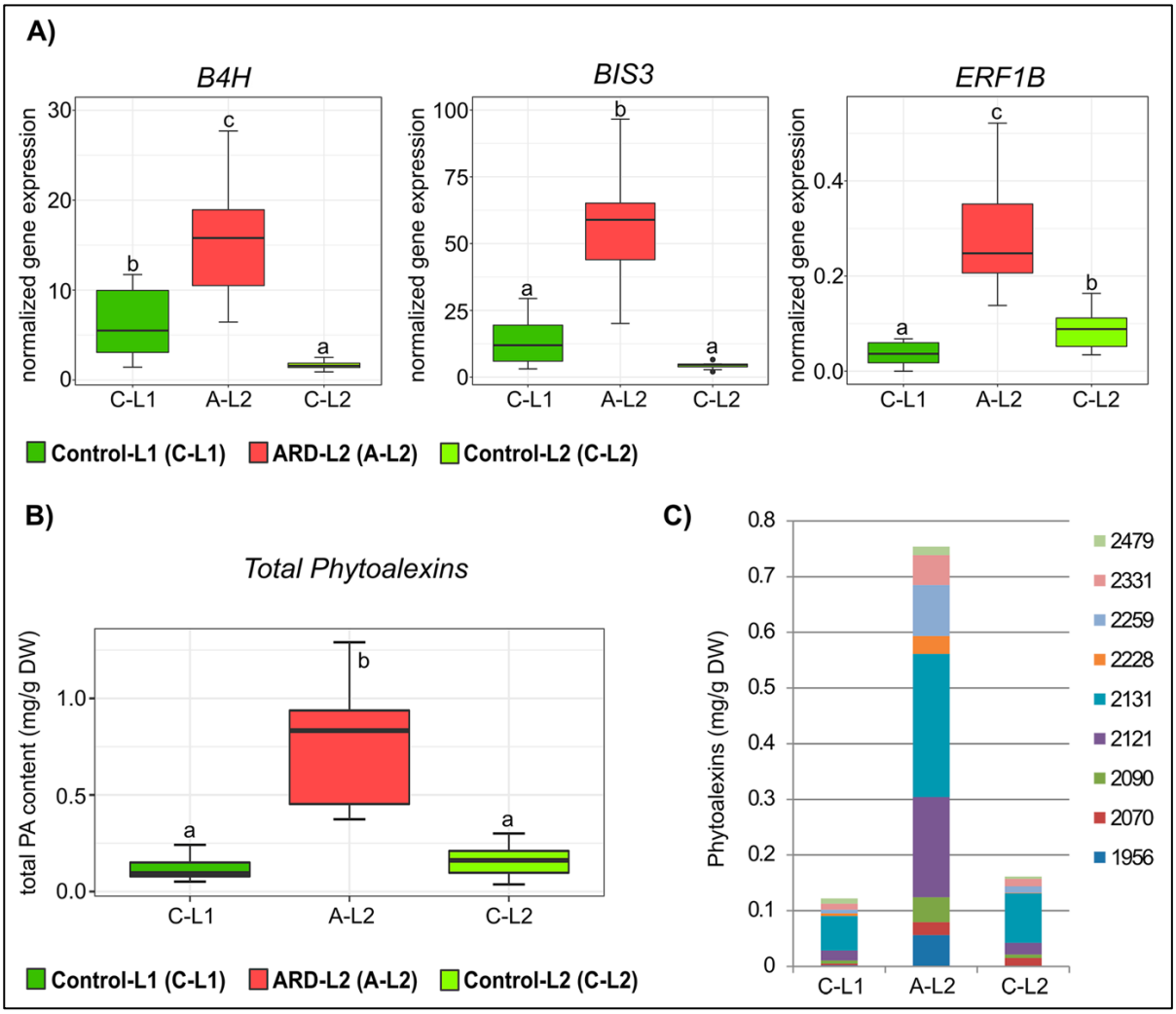

Figure 3. Analysis of ARD biomarker genes and phytoalexins in roots of apple genotype M26 grown in columns for 29 days. (A) Normalized expression of the ARD biomarker genes biphenyl synthase 3 (BIS3), biphenyl 4-hydroxylase (B4Hb) and ethylene-responsive transcription factor 1B-like (ERF1B) in roots of 'M26' plants at the positions L1 (Control soil), L2 (ARD soil) and L2 (Control soil) as depicted in Fig. 1. (B) Total phytoalexin content in Control and ARD soils under fCT and sCT scanfrequencies. (C) Levels of individual phytoalexins identified by GC-MS. Compounds are indicated in the order of increasing retention index (RI). 1956, 3-hydroxy-5-methoxybiphenyl; 2070, isomer of noraucuparin; 2090, aucuparin; 2121, noraucuparin; 2131, 2-hydroxy-4-methoxydibenzofuran; 2228, eriobofuran; 2259, noreriobofuran; 2331, hydroxyeriobofuran; 2479, 3,9-dimethoxy-2,4-dihydroxydibenzofuran. Different letters indicate a statistical difference between variants as assessed by multiple comparisons (Tukey test, $P < 0.05$; $n = 10$).

Normalized gene expressions were all significantly higher for ARD-L2 roots compared to adjacent Control-L1 and -L2 (Fig. 3A). For *BIS3*, the difference was most pronounced, with a fold-change increase of 3.9 (ARD-L2 *vs.* Control-L1) and 12.9 (ARD-L2 *vs.* Control-L2). *B4H*

also showed an increased expression fold change of 2.5 (ARD-L2 *vs.* Control-L1) and 9.7 (ARD-L2 *vs.* Control-L2), while fold changes for *ERF1B* were 6.7 and 3.1, respectively.

Gene expression differed between the two control compartments with a higher expression of *BIS3* and *B4H* in the Control-L1 (significant for *B4H* and only in tendency for *BIS3,* Fig. 3A). In contrast, transcription factor *ERF1B* expression was significantly higher in Control-L2 compared to Control-L1 (Fig. 3A). The expression of the phytoalexin biosynthesis genes *BIS3* and *B4H* was higher compared to the transcription factor *ERF1B*.

3.3 Phytoalexin content in roots

There were no significant differences in root phytoalexin amounts between CT-scan frequencies (Fig. S4, Supporting Information). The root phytoalexin content in ARD-L2 (0.76 ± 0.25 mg/g DW) was 6.3 and 4.5 times higher than in Control-L1 (0.12 ± 0.06 mg/g DW) and Control-L2 (0.17 ± 0.08 mg/g DW), respectively (Fig. 3B). Between Control-L1 and Control-L2, the phytoalexin contents did not statistically differ. Identified compounds included four biphenyls and five dibenzofurans (Fig. 3C), the majority of which being present at low levels in the two controls. The top three phytoalexins were 2-hydroxy-4-methoxydibenzofuran, noraucuparin and noreriobofuran. 2-Hydroxy-4-methoxydibenzofuran was detected in all samples and accounted for > 50 % of the total phytoalexin content in Control roots. In ARD-L2 roots, the 2-hydroxy-4-methoxydibenzofuran levels were increased 4.1 and 2.9 times compared to Control-L1 and -L2, respectively. The 3-hydroxy-5-methoxybiphenyl content in ARD-L2 was even increased to 0.06 mg/g DW, being 69 times higher than in Controls. Considerable variation was observed in the levels of individual compounds among replicates in both ARD and Control soils.

3.4 Soil layer, substrate, and CT scan frequency effects on microbial communities

748,965 high-quality 16S rRNA gene sequences (8,607–23,925 per sample) and 1,531 OTUs were recovered for bacteria and archaea. For fungi, 1,360,631 ITS2 sequences (6,476–50,922 per sample) and 2,361 OTUs were obtained.

3.4.1 Alpha-diversity analysis

For bacterial/archaeal profiles, significantly lower richness (species richness and ACE) and evenness (Simpson and Shannon) in the rhizoplane compared to bulk soil was detected (Fig. 4). For fungi, the same trend was observed for richness (species richness and ACE, Fig. 4) but no differences were observed for their evenness.

For bacterial/archaeal profiles, significant but minor effects of CT-scan frequencies were detected for species richness and ACE, being higher for sCT (Table S4, Supporting Information). For fungi, no effect of CT-scan frequencies was observed (Table S4, Supporting Information). As effects of other factors were important and highly significant, we decided to average fCT and sCT values (n = 7-10, Fig. 4). Rhizoplane bacteria/archaea diversity indices were significantly lower for richness (species richness and ACE) and evenness (Simpson and Shannon) in ARD-L2 compared to Control-L1 and -L2. For fungi, the same trend was observed for richness in rhizoplane samples, while the opposite was observed for bulk soil samples.

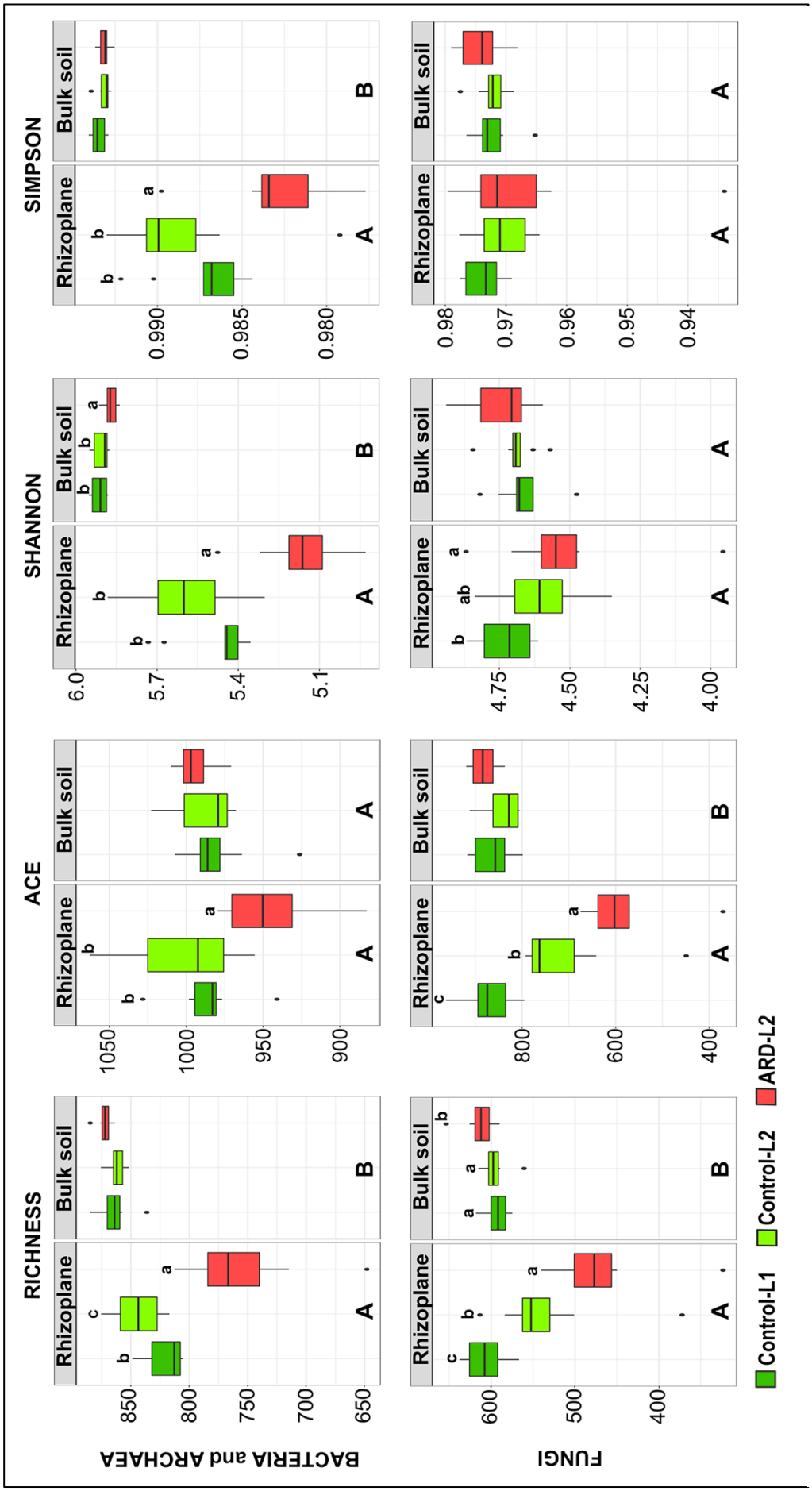

Figure 4. Microbial alpha-diversity analysis (n = 7-10). Alpha-diversity of the bacterial/archaeal and fungal communities for each substrate (Control, ARD) across the different microhabitats (rhizoplane, bulk soil) and soil depths (layer 1/L1 and layer 2/L2). Box plots display the following alpha-diversity indices calculated from 16S rRNA gene and ITS-2 fragment amplicon sequencing data: Species richness, ACE, Shannon and Simpson. Samples below our quality thresholds were not included in this analysis (Table S3), leaving between 7 and 10 replicates per treatment. Different lowercase letters and uppercase letters represent statistically significant differences between substrates within each microhabitat and between microhabitats, respectively (ANOVA, post-hoc Tukey s HSD correction test). Whiskers are coloured by sample type.

3.4.2 Beta-diversity analysis

First, we tested if the sampling inside the same experimental unit (column) had an effect on the microbial community composition (column effect). No significant column effect on the microbial community composition was identified ($P > 0.05$). In terms of community structure, no effects of CT-scan frequencies were detected on bacterial/archaeal ($P = 0.13$, Fig. S5, Supporting Information) and fungal profiles ($P = 0.07$, Fig. S6, Supporting Information). Significant effects of microhabitats, soil substrate and their interaction were detected for both bacterial/archaeal (Fig. S5, Supporting Information) and fungal profiles (Fig. S6, Supporting Information). For bacterial/archaeal profiles, the main factor was the difference between microhabitat (bulk soil *vs.* rhizoplane, CAP1 = 50.6 %) followed by ARD (Control soil *vs.* ARD soil, CAP2 = 14.3 %). For fungal profiles, the main factor was ARD (Control soil *vs.* ARD soil, CAP1 = 34.3 %), followed by microhabitats (bulk soil *vs.* rhizoplane, CAP2 = 11.3 %). The microhabitat effect was clear at phylum level (Table S5, Supporting Information) with an increase of Proteobacteria and Candidatus Saccharibacteria in TC-DNA from rhizoplane samples, while only the endomycorrhizal Glomeromycota and Olpidiomycota were increased for fungi. The ARD effect was seen at phylum level for both bacteria/archaea and fungi (Table S6 and Table S7 respectively, Supporting Information) compared to Controls. Gemmatimonadetes showed a significant increase in ARD-L2 compared to Controls in both microhabitats (rhizoplane and bulk soil; Table S6, Supporting Information). In the rhizoplane, Betaproteobacteria were increased in ARD-L2 (14 %) compared to Controls (7-9 %). In bulk soils, Firmicutes, Gammaproteobacteria and Gemmatimonadetes were increased in ARD-L2 compared to Controls. Conversely, Deltaproteobacteria, Acidobacteria and Latescibacteria (WS3) were increased in Controls samples compared to ARD-L2 in all microhabitats. Verrucomicrobia, Actinobacteria and Nitrospirae were increased in Controls of bulk soils. For fungi, a significant increase of Rozellomycota, Mucoromycota, Glomeromycota and Olpidiomycota was detected in Controls compared to ARD-L2 (Table S7, Supporting

Information). Mortierellomycota were increased in Control bulk soils only. Conversely, only Ascomycota were increased in ARD-L2 samples compared to Controls in all microhabitats. For all microbial profiles, the significant interaction between soil substrate and microhabitat was due to notable differences between rhizoplane communities in Control-L1 and -L2, no longer seen in bulk soils (Fig. S5-S6, Supporting Information).

A refined analysis was made separately in the bulk and the rhizoplane samples for all microbial groups (Fig. 5), revealing a significant difference between microbial communities in Control and ARD substrates, being exacerbated in bulk soils (CAP1 = 56-62 %) compared to rhizoplanes (CAP1 = 27-33 %). Rhizoplane differences between Control soils in L1 and L2 were minor (CAP2 = 8-14 %), and marginal in bulk soils (CAP2 = 3 %). The peculiar community structure identified in ARD exposed roots is also coherently associated with the shorter root length observed in these specific samples, be it for the fungi in the rhizoplane (R^2 = 0.15, P = 0.001) and bulk soil (R^2 = 0.23, P = 0.001), as well as for the bacteria and archaea in the rhizoplane (R^2 = 0.21, P = 0.001) and bulk soil (R^2 = 0.19, P = 0.001).

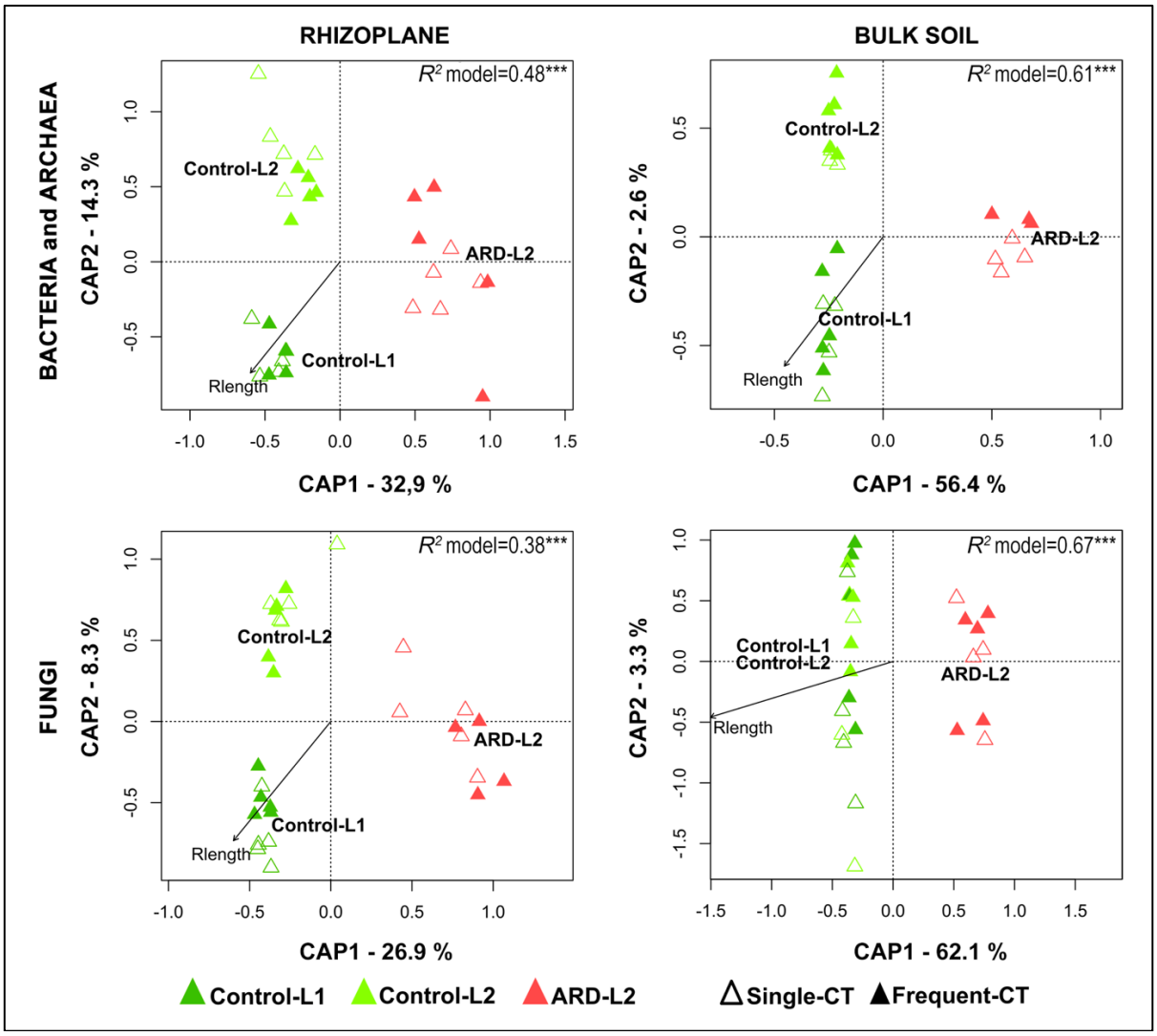

Figure 5. Microbial beta-diversity analysis (n = 7-10). Distance-based redundancy analysis of bacterial/archaeal and fungal communities for each substrate (Control, ARD), depth (layer 1/L1 and layer 2/L2) and CT frequency (frequent CT/fCT, single CT/sCT) across microhabitats (rhizoplane and bulk soil). Analysis done on log10 transformed 16S rRNA gene and ITS-2 fragment amplicon sequencing data (Bray-Curtis dissimilarity index, 9999 permutations). Percentage of variance explained (R-square or R2) and significance (ANOVA) of the model shown. The level of significance is displayed with stars: (∗) 0.05 ≤ P-values < 0.01; (∗∗) 0.01 ≤ P-values< 0.001; (∗∗∗) P-values ≤ 0.001. Root length data was added to display its correlation with microbial community composition.

3.5 Identification of microbial OTUs responding to substrate

"Responders" or OTU taxa that were significantly enriched or depleted in ARD-L2 samples in comparison to Control-L2 samples were identified. A total of 244 bacterial/archaeal and 134 fungal rhizoplane responders were found. Dominant responders were displayed in a heatmap

(Fig. 6). Numerous dominant Actinobacteria (*Streptomyces*) Alpha- (e.g. *Novosphingobium*, *Sphingobium*, *Sphingomonas*, *Asticcacaulis* and *Bosea*), Beta- (*Burkholderia*, *Variovorax* and Methylophilaceae), and Gammaproteobacteria (e.g. *Pseudoxanthomonas*, *Rhodanobacter* and *Lysobacter*) OTUs were increased in ARD-L2 rhizoplanes (Fig. 6). In Control-L2, phylogenetically diverse OTUs were higher in relative abundance compared to ARD-L2, including the dominant Candidatus Saccharibacteria, *Massilia* and Acidobacteria (*Gp6*).

Several fungal OTUs were assigned only at order level, while others were not even classified at phylum level. In both microhabitats, dominant responding OTUs belonged mainly to Ascomycota, and also Morteriellomycota, Rozellomycota, Mucuromycota and Basidiomycota. Diverse OTUs belonging to classes, i.e. Eurotiomycetes (Herpotrichiellaceae), Rozellomycota, Mucuromycota and Morteriellomycota, were increased in the rhizoplane from roots exposed to Control-L2 compared to ARD-L2 (Fig. 6). Conversely, dominant rhizoplane OTUs increased in ARD-L2 belonged to Pleosporales (Dothideomycetes; ARD: 6 % *vs.* Control: 2 %), Xylariales (Sordiaromycetes), Helotiales (Leotiomycetes) and *Apiotrichum xylopini* (Tremellomycetes). OTUs affiliated to classes Leotiomycetes and Tremellomycetes were exclusively enriched in ARD-L2. *Ilyonectria robusta* (OTU1509) and *Nectria* sp. (OTU1224), potential ARD pathogens, were significantly enriched in ARD-L2 compared to Control-L2 in both microhabitats.

A total of 416 bacterial/archaeal and 350 fungal ARD bulk soil responders were identified. Dominant responders were displayed in a heatmap (Fig. S7, Supporting Information). Several taxa increased in ARD-L2 bulk soil belonged to Acidobacteria (e.g. *Gp4*), Firmicutes (e.g. *Bacillus*) and Thaumarchaeota (*Nitrososphaera*). Conversely, *Gp6* (Acidobacteria) were increased in Controls.

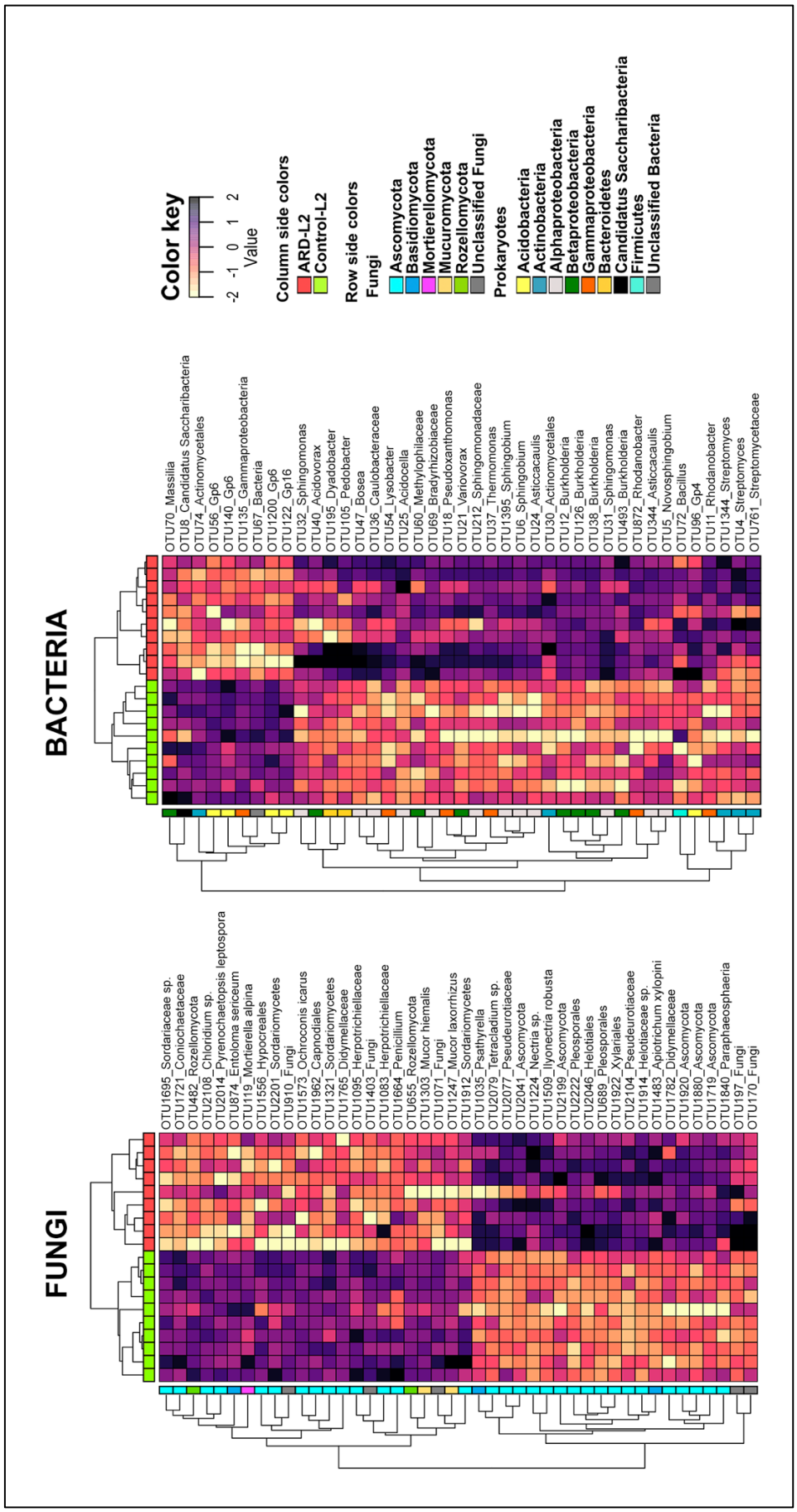

Figure 6. ARD rhizoplane responders (n=9-10). Heatmaps showing bacterial/archaeal and fungal OTUs whose relative abundance is significantly higher or lower in ARD-L2 in comparison to Control-L2 samples in the Rhizoplane (>0.2%). Taxa relative abundance was $\log_{10}$ transformed and center-scaled for the heatmap representation (z-score). Taxa are classified at the species level or the lowest rank possible with the RDP and UNITE database for bacteria/archaea and fungi, respectively. Similarities in community composition between samples determine the top hierarchical clustering (Bray-Curtis dissimilarity index) while similarities between taxa abundance behavior, along the samples studied, determine the side hierarchical clustering. Column-side and row-side colors indicate sample type and taxonomic affiliation at the Phylum level (Class for Proteobacteria), respectively. Significant differences in relative abundance between treatments was determined by negative binomial GLM fit with a likelihood ratio test and corrected using false discovery rate for multiple comparisons (FDR, $P < 0.05$).

In bulk soil, the most abundant fungal OTUs in ARD-L2 belonged to Pleosporales (ARD: 9 % *vs.* Control: 2 %, Dothideomycetes), *Pseudaleuria* (Pezizomycetes), *Solicoccozyma* (Tremellomycetes), Xylariales (Sordariomycetes), *Morteriella* (Morterielloycetes) and Helotiales (Leotiomycetes). Fungi affiliated to the Herpotrichiellaceae (Eurotiomycetes), Mucuromycota and Rozellomycota were uniquely enriched in Control-L2.

The distribution of microbial responders across microhabitats was synthesized in Venn diagrams (Fig. S8, Supporting Information). A high number of responders was uniquely detected in the bulk soil (52-71%). Important overlaps for bacterial/archaeal and fungi responders were detected between rhizoplanes and bulk soils (20-31 %). Ten fungal OTUs were only enriched in rhizoplanes of plants grown in ARD, being absent in bulk soils.

4 DISCUSSION

In this study, we investigated the ability of ARD-causing agents to spread in soil by means of an integrated approach targeting, for the first time, four different levels: root architecture, soil and rhizoplane-associated microbial communities, plant ARD marker gene expression and phytoalexin contents. An innovative experimental design relying on a barrier-free split-root experiment under composite soil with different layers (Control/ARD) was used allowing microorganisms to move freely throughout the soil (Fig. 1). We predict that if ARD-causing agents do not spread through the soil, apple roots grown in the ARD patch will be affected by the disease while roots in Control soils will develop normally.

4.1 Local root growth reduction and strong biotic stress defense response in ARD-exposed roots.

Despite water movements in our soil column and lack of physical barrier, we only observed root growth reductions in roots of plants grown in ARD soil patches. This is a major experimental evidence confirming the local occurrence of ARD symptoms (Lucas *et al*. 2018; Balbín-Suárez *et al*. 2020). We demonstrated for the first time that not only root growth was arrested locally by ARD soil but also the plant defenses, i.e. expression of ARD indicator genes and total phytoalexin contents, were significantly higher in roots exposed to ARD soil. No systemic induction of ARD indicator genes was detected confirming the local and specific response aspect (Lucas *et al*. 2018; Reim *et al*. 2020; Rohr *et al*. 2020). However, our study was not designed to identify a subsequent systemic reaction that might be triggered later (Henfrey, Baab and Schmitz *et al*. 2015; Weiß and Winkelmann 2017). Indeed, phytoalexin formation (especially biphenyls and dibenzofurans) is a plant defense strategy against pathogen invasion (Chizzali and Beerhues 2012), creating a local environment that inhibits microbial growth and propagation, prior to systemic resistance induction (Tian and Zhang, 2019). This "phytoalexin signature" is reasonably orientated toward a microbial origin, be it from the original ARD-specific soil or the later root-associated microbiome. Deeper analysis relying on strain isolation and application as well as specific effects of phytoalexin compounds on root microbiome would be useful.

4.2 Local alterations of root-associated microbiome (dysbiosis) and higher abundance of potential ARD pathogens in ARD-exposed roots

Dysbiosis is defined as any deviation in community structure of the resident root/gut microbiome of healthy plants/individuals that is linked with host disease (Petersen and Round 2014; Mendes and Raaijmakers 2015; Smets and Koskella 2020). These changes might be crucial in ARD disease development, as the presence of a disrupted root-associated microbiome might facilitate pathogen establishment (Mendes *et al*. 2011) or trigger the disease itself by altering adequate host immune responses, like those observed for human gut diseases (Petersen

and Round 2014). Previous split-root analysis revealed the existence of an altered bacterial and archaeal community in ARD bulk soils and rhizoplane samples (dysbiosis), both in terms of alpha and beta-diversity (Lucas *et al*. 2018; Balbín-Suárez *et al*. 2020). Here we confirm this finding with regard to bacteria/archaea, and broaden it to the fungal community for the first time.

Indeed, our analysis revealed a significantly reduced microbial richness (bacteria/archaea and fungi) and evenness (bacteria/archaea) in ARD-affected rhizoplanes, a characteristic previously related to facilitation of pathogen establishment or microbial invasions (Mallon, Van Elsas and Salles 2015; Yang *et al*. 2017). Likewise, bacterial/archaeal and fungal community compositions were profoundly altered in ARD-affected samples across microhabitats.

Potential fungal pathogens associated with ARD, like *Rhizoctonia* (Mazzola 1997) and *Fusarium* (Franke-Whittle *et al*. 2015; Manici, Caputo and Saccà 2017), were either not detected or were not significantly enriched in ARD (< 0.02 %). However, members of the Pleosporales, Helotiales and Nectriaceae (*Ilyonectria robusta* and *Nectria* sp.) that were significantly enriched in ARD rhizoplanes and bulk soils, were previously found to be associated with ARD soils, isolated from ARD-exposed roots and proved pathogenic towards apple (Deakin *et al*. 2018; Manici *et al*. 2018; Popp, Grunewaldt-Stöcker and Maiss 2019; Popp *et al*. 2020). The presence of other potential ARD pathogens like *Pythium* sp. and *Phytophthora* sp. could not be addressed in the present study, as the ITS primers used did not target oomycetes (Mazzola *et al*. 2002; Tewoldemedhin *et al*. 2011).

Bacterial taxa differentially enriched in rhizoplane/rhizosphere samples from ARD exposed roots (e.g. *Variovorax*, *Streptomyces*, Methylophilaceae) or Control samples (e.g. *Spartobacteria*) were consistent with previous studies (Mazzola 1999; Tilston *et al*. 2018; Balbín-Suárez *et al*. 2020). Interestingly, ribotypes affiliated to *Streptomyces* were found to be increased in abundance also inside the roots of ARD-affected plants, suggesting their

involvement in ARD development (Mahnkopp-Dirks *et al*. 2020). Moreover, several taxa known for their aromatic compound degradation capabilities like *Burkholderia* (Krastanov, Alexieva and Yemendzhiev 2013)*, Sphingobium, Streptomyces* (Seo, Keum and Li 2009)*, Sphingomonas* (Zhao *et al*. 2008)*, Variovorax* (Satola, Wübbeler and Steinbüchel 2013) and the fungal genus *Apriotrichum* (Mašínová *et al.* 2016) were consistently enriched in ARD rhizoplane samples, being concomitant with the presence of higher concentration of phenolic compounds in ARD-affected roots (Henfrey, Baab and Schmitz 2015; Weiß *et al*. 2017; this study). This would support the notion that the rhizoplane dysbiosis state may be an indirect consequence of plant responses to ARD. Functional speculation on taxonomic results should be taken with care, as another study, based on metagenome analysis, indicated a reduced potential for degradation of phenolic compounds by rhizosphere microbes under ARD (Radl *et al*. 2019).

Overall, our observations and previous findings (Yim, Smalla and Winkelmann 2013; Sun *et al*. 2014; Balbín-Suárez *et al*. 2020), point toward a systematic alteration of microbial communities in ARD soil and the subsequent root-associated microbiome developed from that same ARD bulk soil. Therefore, our findings can be interpreted as evidence for a dysbiosis state of rhizoplane microbiome that was observed only on ARD-exposed roots, thus reinforcing the local response assumption. It is still not fully understood whether a dysbiotic microbiome is directly associated with host-disease susceptibility, or whether it is an indirect consequence of the disease itself (Bäckhed *et al*. 2012; Belizário and Napolitano 2015). Further studies are required to elucidate whether this dysbiotic microbiome is a common ARD feature (across sites and rootstocks), and a cause or a consequence of the disease.

No significant effects of the CT-frequency were observed, except for minor effects on the bacterial and archaeal communities, whose richness was slightly decreased with repeated scans. This is in agreement with Ganther *et al*. (2020), who found no (microbial community

composition) or only transient (gene expression) changes upon X-ray CT exposure (using similar X-ray doses and the same geometry).

4.3 Does ARD spread through the soil?

The deliberate absence of physical barriers in our split-root design allowed testing the lack of horizontal spreading in ARD under controlled conditions. We concluded that ARD-causing agents have most likely a low mobility and do not spread through soil, at least not in the short term (28-day experiment), as only roots in contact with ARD soil were affected by the disease showing: i) local root growth reductions and enhancement of plant defense reactions (phytoalexins), ii) an altered root-associated microbiome (dysbiosis) and iii) a significant enhancement of potential ARD-causing agents abundance. The lowered microbial diffusion potential observed between ARD-L2 and Control-L2 neighbor compartments reinforced as well the aspect of the low mobility of the ARD-causing agents. ARD-causing agents' lack of spreading through soil could be as well explained by their inability to increase in abundance under specific microbial contexts. Indeed, other studies reported that aggressiveness or pathogenicity of potential ARD pathogens was often reduced when inoculated in commercial or grass soils in contrast to sterile soils (Mazzola 1997; Manici, Caputo and Saccà 2017; Popp *et al*. 2019). Hence, pathogen virulence and dispersal abilities may be influenced by the presence of specific microbial assemblies (e.g. dysbiosis) or microorganisms (e.g. synergistic effects) that may facilitate or even promote pathogen attack as already suggested by Manici *et al*. (2018). However, our 28-day experiment might not have been sufficient for pathogens with low growth rates, like *Ilyonectria* sp., to efficiently spread throughout the soil (Manici *et al*. 2018). Further studies need to be conducted to confirm the findings of the present study, including additional soil textures and plant genotypes.

4.4 Are apple plants able to sense ARD soil?

The sessile nature of plants forced their acquisition of fine mechanisms to sense environmental changes to appropriately adjust their physiology, e.g. tropisms or directional plant organ growth movements (Esmon, Pedmale and Liscum 2005; Muthert *et al*. 2020).

In the present study, we tested the apple plant's ability to sense ARD soil presence by providing a choice of substrate for root growth. While high root lengths were observed in the Control layers (L1 and L2), only a small proportion of roots originated from the ARD-L2 soil. The root growth kinetic observed via frequent scanning showed head-on growth through ARD-L2, followed by marked growth retardation. Thus, it can be concluded that a direct contact is required with ARD soil to trigger root growth inhibition. If volatiles are involved, their action range will not exceed few centimeters at best. Thus, the most parsimonious explanation would be that direct exposition to causing agent(s) is required. If avoidance is defined *sensu stricto* as no growth into the ARD substrate and/or altered root negative tropism, we should rule out the existence of apple plant negative tropisms towards ARD soil. However, if avoidance is defined *sensu largo* at the entire root system level, negative tropism may still be valid. Indeed, the share of roots found in each respective compartment, *i.e.* root system plasticity (Hodge 2006), will depend on growth conditions in the neighboring compartment (Control soil) and will be also influenced by root gravity tropisms (Kiss 2007). Further studies of apple root negative tropism towards ARD soil are required to unravel the root-sensing mechanism, the vector nature (e.g. microbes or molecules), and the route (water, volatile).

5 CONCLUSION

Our barrier-free column split-root experiment supports the idea of ARD-causing agents not spreading through soil, as only apple roots exposed to ARD soil were affected. This was evidenced by lower root length, different diameter distribution, enhanced expression of ARD indicator genes and phytoalexin contents and rhizoplane microbiome shifts. Apple plant roots growing straight into ARD soil will display the symptoms, without evidence supporting avoidance by altered tropisms. Alpha and beta-diversity indicated a strong dysbiosis of the global microbial community in the rhizoplane of ARD roots that might be caused by the exposure to an already deeply altered microbiome in ARD bulk soils, together with the strong local plant defense response. Potential apple plant pathogens (Pleosporales, Helotiales, *Ilyonectria*) in rhizoplane and/or in bulk soil samples might have triggered the strong local plant defense response and the subsequent root accumulation of phenolic compounds (phytoalexins) and recruitment of phenolic microbial degraders (e.g. *Variovorax*, *Streptomyces*, *Apiotrichum*). The rhizoplane dysbiosis might be, therefore, a consequence of ARD or part of ARD disease development. Further microbiome studies focusing on the restoration of a healthy apple rhizosphere/rhizoplane microbiome (rebiosis), e.g. by increasing microbial diversity and/or activation of its indigenous suppressive potential (through organic amendments or inoculants), could open new perspectives for the development of more sustainable ARD counteraction strategies.

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Conflict of interest statement

The authors declared that there is no conflict of interest.

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6 General discussion

In the following chapter, the findings of this thesis are collectively discussed. Firstly, possible reasons for the shoot stunting caused by ARD are connected to the findings of this thesis in chapter 6.1. We found that plants to some degree compensate vegetative growth when partly exposed to healthy soil and discuss possible causes and implications thereof on ARD severity assessment in chapter 6.2. In chapter 6.3 unspecific reactions of the ARD biomarker genes are discussed given the complexity of ARD. With the prominent upregulation of phytoalexin biosynthesis in ARD, the role of phytoalexin composition and total amount for ARD etiology is discussed in chapter 6.4. Chapter 6.5 focuses of the possible contribution of phytoalexins and additional phenolic compounds in ARD development. Finally, an outlook is presented in chapter 6.6.

6.1 Shoot stunting caused by ARD as a possible result of water limitation triggered by root damage

Within this thesis, the three genes *biphenyl synthase 3* (*BIS3*), *biphenyl 4-hydroxylase b* (*B4Hb*) and *ethylene-responsive transcription factor 1B-like* (*ERF1B*) were identified as transcriptional indicators for ARD from a set of previously identified candidate genes. Taken all ARD gene expression studies into account, this conclusion is based on eight temporally independent experiments and six ARD soils of different origins (Weiß et al., 2017a; Weiß and Winkelmann, 2017; Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020; Rohr et al., under review; Balbín-Suárez et al., in press). Plant growth varied quite strongly between the different soil types used with the strongest ARD symptoms of growth reduction and root damage visible in the sandy soil from Heidgraben (used in Mahnkopp et al., 2018; Reim et al., 2020; Rohr et al., 2020; Rohr et al., under review) and soil K (Weiß et al., 2017b). It has been discussed previously that ARD is more easily induced in sandy soils (Mahnkopp et al., 2018; Winkelmann et al., 2019; Reim et al., 2020). ARD symptom severity may be depending on the respective soil's capability to suppress pathogens. Garbeva et al. (2004) refer to this as soil suppressiveness, which is believed to be linked to the content of soil organic matter as well as the presence of specific biocontrol microorganism communities. Furthermore, soil water holding capacity is decreasing with increasing sand content.

One hypothesis of Reim et al. (2020) was that changes in auxin, cytokinin, abscisic acid, and gibberellin homeostasis and signaling are expected to occur in shoots of ARD affected plants

explaining the strongly altered shoot morphology. Even though expression of the ARD biomarker genes *BIS3* and *B4Hb* was negatively correlated with shoot growth, root-shoot communication under ARD conditions still has to be identified (Weiß and Winkelmann, 2017; Reim et al., 2020). In the roots, Zhu et al. (2014) and Shin et al. (2016) found genes induced involved in phytohormone signaling upon *Pythium ultimum* infection. Furthermore, the authors showed ethylene, salicylic and jasmonic acid biosynthesis and signaling genes induced as part of the biotic defense response. Further comparative studies in apple roots and aboveground parts could shed further light on the transcriptomic hormonal changes in the shoot. The stunting could also be explained without a prominent regulatory involvement of plant growth hormones in the shoot. It could be a result of the root damage caused by ARD causal agents limiting water uptake. A limited nitrogen uptake has already been found for ARD affected roots (Lucas et al., 2018). Thus, the enhanced stunting observed in sandy ARD soils may be a combination of reduced soil suppressiveness and growth limitation by hindered water uptake.

6.2 Vegetative growth compensation causes uneven habitus in the field and limits biotests solely based on growth parameters

In the field, ARD is characterized by stunting and uneven growth (reviewed in Mazzola and Manici, 2012 and Winkelmann et al., 2019). The results of our split-root and split-column experiments (Rohr et al., under review; Balbín-Suárez et al., in press) shed more light on possible reasons of this observation. It is known that ARD causal agents are largely immobile in the soil and thus, as the plants explore the soil they are likely faced with both healthy and ARD soil simultaneously. There is now substantial evidence that plants respond to ARD in a highly localized manner. ARD causes alterations in root morphology and growth, marker gene expression and contents of phytoalexins and other polyphenolic compounds. These alterations are caused by a direct or very close exposition to the ARD microbiome and potential involved volatiles (Lucas et al., 2018; Rohr et al., under review; Balbín-Suárez et al., in press).
It seems that this targeted response in the roots may allow the plants to save resources (discussed in Zhu and Saltzgiver, 2020) and compensate vegetative shoot growth depressions as seen in three split-root experiments (Lucas et al., 2018; Rohr et al., under review). The inhomogeneous shoot growth is thus a result of an uneven distribution of ARD causal agents in the soil and the plants' capability of partial compensation. This is supported by dilution experiments where

mixing of ARD soil with 20 – 95 % healthy soil without spatial separation did not yield proportionally strong effects on ARD symptom reduction (Hoestra, 1968; Jaffee et al., 1982; van Schoor et al., 2009; Tewoldemedhin et al., 2011b; Spath et al., 2015).

Overall, shoot growth is influenced by a variety of factors, which limits reliability of growth-based biotest and highlights the usefulness of other early indication systems for ARD. An advantage of the biotest developed by Yim et al. (2013) is the option of a non-destructive measurement of the deciding parameter, i.e. shoot length. More reliable alternatives like light microscopy of ARD symptoms (Grunewaldt-Stöcker et al., 2019) or analysis of gene expression (e.g. Shin et al., 2016; Zhu et al., 2016; Weiß et al., 2017a; Weiß et al., 2017b; Reim et al., 2020) are destructive in nature as they are focused on root material. On the other hand, these analyses are up to current knowledge most reliable as there is now substantial evidence that apple plants focus their response to ARD in the roots (Rohr et al., under review). Such analyses entail a higher investment in technology but also necessary expertise, which yields more precise results while shoot length measurements are performed easily and comparably cheap. This makes the current options a trade-off between input of resources and reliability of the diagnosis. A combination of several methods is presently the most reliable approach to diagnose ARD and understand factors influencing its development and severity.

For a wider use of the identified transcriptional ARD markers, their accessibility would need to be improved. Two plausible improvements would be an increase in sample throughput and simplification in methodology. Sample throughput could be increased by the use of high throughput qPCR systems like the BioMark HD used by Reim et al. (2020) or hybridization-based systems like microarrays. Alternatively, phytoalexin compounds like 3-hydroxy-5-methoxybiphenyl could be analyzed, since their abundance was strongly positively correlated with the expression of *BIS3* and *B4Hb*. This would lead to a slight simplification in terms of sample handling and storage without the dependency on RNA. However, the methodology of gas chromatography and mass spectrometry, which also allows for a high sample throughput, is similarly intricate in execution as gene expression studies. Future studies could shed more light on the suitability of the BIS3 and B4Hb proteins as possible ARD indicators, which may enable the development of an immunoassay such as an enzyme-linked immunosorbent assay (ELISA).

6.3 The complexity of ARD and diversity of rootstock genotypes makes the identification of an unbiased biomarker challenging

The biphenyl synthases *BIS1* to *BIS4* and the biphenyl 4-hydroxylases *B4Ha* and *B4Hb* showed a strikingly high regulation amongst the 108 candidate genes investigated in the roots of four apple genotypes growing in two ARD soils (Reim et al., 2020). By far the highest transcript level was present for *BIS3* in the roots, which along with *B4Hb* was found to be early and consistently upregulated in additional ARD soils but unaffected by abiotic stressors (Rohr et al., 2020). An upregulation of these genes was observed in grass soil after one week as well, which remained at a moderate level after two weeks while the response in ARD soil was further boosted (Rohr et al., 2020). The third candidate gene tested, *ethylene-responsive transcription factor 1B-like* (*ERF1B*), also showed a consistent regulation in the three ARD soils but also under heat stress. Since ERF transcription factors play a prominent role in the general stress response (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Oñate-Sánchez and Singh, 2002), cross reactions were to be expected.

Although no strong regulation of *BIS3* has been found in leaf material under ARD conditions (Weiß and Winkelmann, 2017), the gene was induced locally in apple shoots upon inoculation with *Erwinia amylovora*, where phytoalexins are involved in limiting the spread of the bacteria and thus disease progression (Chizzali et al., 2012a; Chizzali et al., 2013). In the roots, infection with *Pythium ultimum* also led to a *BIS3* upregulation (Shin et al., 2016; Zhu et al., 2016). While the former is not a part of the ARD complex, *Pythium* is one of the ARD key causal agents, indicating that also phytoalexins are involved in a more general biotic stress response. Testing the response of the ARD biomarker genes with microorganisms both not associated with ARD and closely associated with ARD would be useful to shed more light on their function in the biotic stress defense and might even enable a differentiation between these organisms in regard to their involvement in ARD etiology.

Because of the observed cross reactions and since ARD comprises a vast number of causal agents, the use of *BIS3* and *B4Hb* as biomarkers always should be accompanied by other indications for ARD such as the growth biotest and evaluation of root morphology. Especially when different apple genotypes are compared i.e. to assess ARD susceptibility, tree vigor has to be taken into consideration by comparing growth in ARD soil to a reference (Deakin et al., 2019). Up to now, only few Malus genotypes have been described as less ARD susceptible, such as *Malus* × *robusta* 5 and offspring thereof (Reim et al., 2019). Reim et al. (2019) tested 51 gene bank accessions of 18 *Malus* wild species in a biotest with untreated and gamma-irradiated

ARD soil and assessed their ARD susceptibility in relation to the rootstock genotypes CG41, CG16, CG41, M26, M9 and the Dresden-Pillnitz rootstock AU56-83. The authors classified the following six *Malus* accessions as less ARD susceptible: *M.* × *robusta* 5 MAL0991, *M. sargentii* MAL0939, *M. baccata* MAL0780, *M.* × *robusta* MAL0595, *M. prunifolia* MAL0407 and *M. spectabilis* MAL0130. Using gene expression analysis coupled with a biotest and microscopic root assessment, the apple genotype B63 (Cummins and Aldwinckle, 1983; Reim et al., 2019) was grouped as ARD sensitive while the ARD tolerance of the *M.* × *robusta* accession MAL0595 was confirmed (Grunewaldt-Stöcker et al., 2019; Reim et al., 2020).

Although crop rotation systems are unfeasible in commercial apple cultivation, a rotation of different rootstock genotypes may be a measure to counter ARD development (Deakin et al., 2019), which the authors attribute to the differential recruitment in rhizosphere microbial recruitment via root exudates relying on genetic factors (Burns et al., 2015; Haichar et al., 2008; St. Laurent et al., 2008; Qin et al., 2016). The current commercial standard for dessert apple cultivation is the highly susceptible dwarf rootstock M9 (Isutsa and Merwin, 2000; Mazzola et al., 2009). Current alternatives, which could be integrated into rotation systems, are generally not well accepted commercially due to an undesirable habitus or genetic incompatibility with the scions (Deakin et al., 2019).

6.4 Total amount and composition of biphenyl and dibenzofuran phytoalexins play a role in ARD etiology and response

Over several experiments, a strong correlation was found between *BIS3* and *B4Hb* expression and the contents of biphenyl and dibenzofuran phytoalexins in apple roots grown in ARD soil (Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020; Balbín-Suárez et al., in press). Expression of *BIS3* and *B4Hb* in apple roots under ARD conditions is a reliable indicator for the de novo synthesis and presence of these compounds. Biphenyl and dibenzofuran phytoalexins have been described as limited to members the subtribe Malinae of the Rosaceae family such as *Malus* and *Pyrus* (Liu et al., 2007; Beerhues and Liu, 2009; Liu et al., 2010; Chizzali and Beerhues, 2012; Sircar et al., 2015). Recently, analyses on Rose Replant Disease (RRD) confirmed that *Rosa corymbifera* 'Laxa' indeed did not form these kinds of phytoalexins (Yim et al., 2020).

A general strong increase in total phytoalexin compound contents is observed in apple roots upon growth in ARD soil. In addition to the total contents, the composition of single phytoalexin compounds also changed in response to the applied stress with an increase in diversity with more diverse biotic challenges (Rohr et al., 2020). 3-hydroxy-5-methoxybiphenyl was predominantly accumulated in roots grown in ARD soil in comparison to all other unspecific variants tested. This compound is of special interest, since it was the only one that was distinguishing the ARD phytoalexin profile from the grass soil's profile (Rohr et al., 2020).
In reference samples of several experiments, 2-hydroxy-4-methoxybiphenyl has been found in low concentrations (Weiß et al., 2017b; Reim et al., 2020; Rohr et al., 2020), which indicates a base level in an unstressed state. 2-hydroxy-4-methoxybiphenyl could therefore be a part of a preformed defense system as it was proposed to exist in apple (Zhu et al., 2017). Such a preformed defense system could complement the local induced defense to allow for a much faster reaction upon infection. In roots of the *Pythium* resistant apple genotype G935, Zhu et al. (2017) found a higher expression of genes coding for pathogen pattern recognition receptors plus subsequent signal transduction, proteins and enzymes of biotic defense hormone biosynthesis and signaling as well as pathogen resistance and secondary metabolism biosynthesis in comparison to the susceptible genotype B9. Zhu and Saltzgiver (2020) discuss the energetic trade-off between a preformed and induced defense. The preformed defense requires a higher investment in the absence of pathogens but allows for a much faster response upon infection, while the induced defense is saving resources in the non-infected state providing more flexibility to respond to other stressors but needs more time to respond upon infection. The importance of a rapid defense response was demonstrated in 'Ottawa 3' × 'robusta 5' crossings in response to *Pythium ultimum* attack, where quickly-spreading root necrosis was observed already 24 h after post inoculation in susceptible genotypes, which was delayed by several days in resistant genotypes by the formation of a physical barrier hindering hyphae growth (Zhu et al., 2018).

Figure 1 depicts the current model of biphenyl and dibenzofuran phytoalexin biosynthesis in apple as proposed by Reim et al. (2020). It shows the proposed positions of the BIS3 and B4Hb proteins, whose coding genes have been identified as ARD biomarkers by Rohr et al. (2020), and the phytoalexin 2-hydroxy-4-methoxydibenzofuran, which has been found in low amounts in reference samples and thus might play a role in a preformed defense system. Furthermore, 3-hydroxy-5-methoxybiphenyl is depicted as the product of several reactions potentially in-

volving BIS3 and caffeic acid 3-O-methyltransferase 1 (OMT1). 3-hydroxy-5-methoxybiphenyl is an interesting compound for future research, since it is induced in roots grown in ARD soil and thus likely part of the induced defense (Rohr et al., 2020; Balbín-Suárez et al., in press).

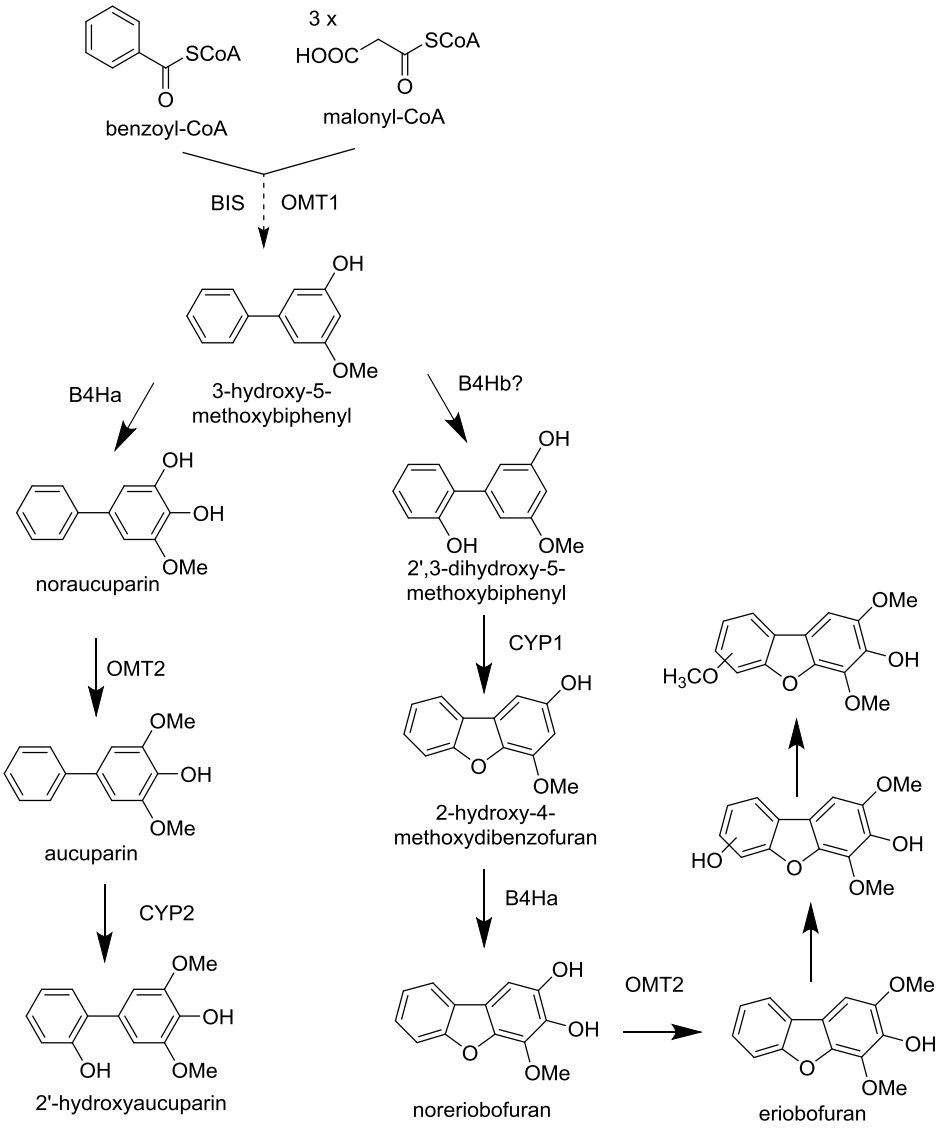

Figure 1: Proposed pathway of biphenyl and dibenzofuran phytoalexins (Reim et al., 2020). Biphenyl synthase (BIS), O-methyltransferase (OMT), biphenyl 4-hydroxylase (B4H), putative cytochrome P450 enzyme (CYP).

6.5 Are biphenyls and dibenzofurans amongst other aromatic compounds released by the roots leading to ARD?

From numerous studies it is now evident, that phytoalexins play an important role in the defensive response of apple against ARD. Furthermore, there is a striking connection between the

narrow spectrum of species affected by replant diseases (especially members of the Rosaceae such as cherry, peach, strawberry, rowan and rose, Winkelmann et al., 2019) and the narrow spectrum of species producing biphenyl and dibenzofuran phytoalexins. Weiß et al. (2017b) proposed harmful effects of these compounds whenever high local concentrations peak in the roots, potentially leading to an autointoxication despite their deemed protective purpose. Although this theory needs further investigation, Weiß et al. (2017b) further hypothesize that ARD sensitive genotypes such as M26 may be defective in phytoalexin detoxification, which less sensitive genotypes are able to overcome. In our study (Reim et al., 2020), we analyzed phytoalexin compounds in more and less sensitive apple genotypes and indeed found a difference: The wild accession and less sensitive genotype *Malus* × *robusta* MAL0595 produced a different phytoalexin composition (absence of eriobofuran and isomers of eriobofuran and noraucuparin) and less total phytoalexins than its sensitive counterparts M26 and B63 (Reim et al., 2020).
Even though possible detoxification mechanisms are not sufficiently explored in apple, phytoalexins were only very specifically induced upon close contact with the diseased soil (Rohr et al., under review; Balbín-Suárez et al., in press). This hints at a possible drawback of these compounds for the plant. Whether this drawback is actual autotoxicity similar to a hypersensitive reaction or an excessive investment for the plant may be the subject of future studies. In situ hybridization studies for phytoalexin biosynthesis genes could for example shed further light on the subcellular localization and triggers of gene expression.

A direct harmful effect of biphenyl and dibenzofuran compounds as the cause for ARD is unlikely, since ARD persists over decades in the soil and turnover of phenolic compounds is much quicker (reviewed in Winkelmann et al., 2019). Moreover, ARD has been attributed to a complex of imbalanced soil biota, including bacteria, fungi, oomycetes, and nematodes (Rumberger et al., 2007; Kanfra et al., 2018), which are causing the soil to lose its ability to sustain the next apple culture (Winkelmann et al., 2019).
With the cause of ARD originating from the apple plant itself, a possible conclusion could be that phytoalexins play a key role in causing the shifts of the soil microbiome leading to ARD. Winkelmann et al. (2019) state that root deposits are responsible for the alterations in the soil microbiome, released either actively by transport or passively via the degradation of root debris. In grass soil, all investigated phytoalexin compounds except 3-hydroxy-5-methoxybiphenyl were most likely induced in response to pathogens native to that soil, since peat substrate did not lead to an induction (Rohr et al., under review; Balbín-Suárez et al., in press). This is a

possible explanation how ARD develops in healthy soil fitting together with the aromatic compound degrading bacterial communities found enriched in ARD soil such as *Burkholderia*, *Sphingobium*, *Streptomyces*, *Sphingomonas*, *Variovorax* and the fungal genus *Apriotrichum* (Radl et al., 2019; Balbín-Suárez et al., in press). The enrichment of these genera could pave the way for other pathogens to manifest in the rhizoplane and rhizosphere such as the ARD causal agents *Cylindrocarpon*, *Phytophthora*, *Pythium*, *Nectriaceae* and *Rhizoctonia*. More studies with grass soil are necessary to further explore this observation.
The enrichment of organism communities specialized surviving under these conditions leads to other (beneficial) organisms being ousted and creating an opening for additional pathogens to thrive. Consequently, the defense reaction of the plant is boosted, which in return leads to a further enrichment of the specialized harmful microorganism communities. Thus, it is plausible that young trees introduced to this situation without an increased defense suffer more than those slowly acclimated to it (Utkhede et al., 1992; Hoestra, 1994).

In rose replant disease (RRD), however, no biphenyl and dibenzofuran phytoalexins have been identified (Yim et al., 2020). Still, cross reactions between apple and rose upon replanting are present, which points at further factors being involved. In rose, phloridzin was found in high concentrations and also under control conditions but in drastically lower abundance compared to apple (Yim et al., 2020). In fact, apple contains by far the highest concentrations of phloridzin and other dihydrochalcones of all members of the Rosaceae (Hofmann et al., 2009; Ogah et al., 2014). Further investigations should focus on a comparison between apple replant disease and replant diseases in other species such as rose to shed further light on shared aspects between them.

6.6 Outlook

Future studies could aim at further characterizing the identified ARD biomarker genes *BIS3* and *B4Hb* by introducing plants to additional abiotic conditions but also to isolated components of the ARD complex. In parallel, common soil microbes not associated with ARD should be tested for the presence and type of a biomarker gene response accompanied by phytoalexin profiling. In situ hybridization techniques could yield information on the gene expression response of *BIS3* and *B4Hb* on a cellular level and answer the question of Balbín-Suárez et al. (in press) at what distance the response is triggered.

The phytoalexin biosynthesis pathway in apple needs to be uncovered completely, which could lead to the identification of further ARD candidate genes. Additionally, phytoalexin exudation and biosynthesis in response to ARD needs further clarification. For this, knock-out lines of *BIS3* and *B4Hb* and in vitro cytotoxicity tests with isolated compounds can be used. First efforts in creating CRISPR/Cas9 knock-out lines have been achieved. At the moment, apple phytoalexins are not commercially available and their extraction at large quantities is a challenge. The group of Ludger Beerhues at the Institute of Pharmaceutical Biology at the Technische Universität Braunschweig is making progress in synthesizing some of these complex compounds.

Growth inhibition tests with pure cultures of ARD causal agents can shed further light on whether these agents are able to survive and / or metabolize phytoalexin and other phenolic compounds such as phloridzin or epicatechin. Metabolization of plant compounds by microbes could for example be tested with isotope labeling. Isolated ARD causal agents have already been tested in soil-independent inoculation systems using apple plants and bacterial and fungal cultures in the joint project of ORDIAmur (Carolin Popp, Felix Mahnkopp-Dirks, Jenny Horn and Nils Orth). Popp et al. (2019, 2020) developed a soil-free test system for ARD causal agents and used Harris Uni-Core punch and laser microdissection to sample ARD associated fungal material for molecular identification. To test the theory of slow enrichment of ARD causal agents in the soil, more timelines of microbiome communities and ARD marker gene expression, phytoalexin profiles would need to be recorded.

Lastly, follow-up studies to Yim et al. (2020) in metabolite and transcriptomic studies in rose under replant conditions may highlight parallels in disease etiology between apple and rose. Since it has been shown that rose lacks the prominent biphenyl and dibenzofuran phytoalexins of apple, comparisons between apple and rose in e.g. transcriptomics can reveal similarities and interactions leading to the observed overlap in the plants' reactions.

7 Conclusions: The buildup of a phenolic defense response involving biphenyl and dibenzofuran phytoalexins could lead to the shift in soil microbiota causing ARD

Phytoalexin biosynthesis plays an important role in ARD as it was revealed by both gene expression studies (amongst others in this thesis) and analyses of biphenyl and dibenzofuran compounds using a set of greenhouse biotests. In contrast to the other candidate genes tested, expression of the phytoalexin biosynthesis genes *BIS3* and *B4Hb* was not impacted by different abiotic stressors and showed an early and consistent upregulation in six ARD soils tested in eight experiments total. Upregulation in gene expression was accompanied by higher amounts of biphenyls and dibenzofurans in the ARD affected roots. This response was found to be induced upon direct or very close contact with ARD soil in two split-root and one split-column experiment and was not systemic. Gene expression and phytoalexin contents were also present in healthy grass soil but to a significantly lesser extent. This and the fact that phytoalexins are very specific compounds produced by *Malus* and *Pyrus* lead to the hypothesis that phytoalexins amongst other polyphenol compounds lead to the formation of ARD via the shift of the soil microbiome community. Comparing apple genotypes with differing sensitivity towards ARD, we found both a lower expression of phytoalexin biosynthesis genes and lower concentrations of biphenyl and dibenzofuran compounds in roots of a less susceptible genotype growing in ARD soil.

But how can a lower concentration of defensive compounds be more effective against a disease? Possibly, the aromatic defense compounds are ineffective, leading to a growing enrichment of detrimental soil microbial communities while the defensive response is further boosted as explained below.

In this theory, apple plants use phytoalexins and other phenolic compounds in the defense against pathogen present in healthy soil. These compounds reach into the soil either actively via rhizodeposition or passively by the degradation of dead root material and lead to a shift in the soil microbial communities. While some microbes are successfully suppressed by the plant's defense, other species such as *Burkholderia*, *Sphingobium*, *Streptomyces*, *Sphingomonas*, *Variovorax* and the fungal genus *Apriotrichum* are able thrive due to their ability to survive, degrade and possibly metabolize these aromatic compounds. This may enable pathogenic microorganisms and causal agents of ARD such as *Cylindrocarpon*, *Phytophthora*, *Pythium*, *Nectria* and *Rhizoctonia* to colonize the rhizosphere and invade the plant. With growing pathogen pressure, the plant in turn responds with an increased defense reaction, which was

seen in a formation and accumulation of phytoalexin compounds in ARD soil in comparison to healthy soil. It is unknown if this way even toxic levels of defensive compounds are reached locally and if further phenolic compounds are involved in the recruitment of harmful soil communities by the apple plant. The old plant is able to survive this increasing pathogen pressure by rising its defenses and growing into unaffected soil regions. The partly growth in healthy soil enables the plants to compensate aboveground growth to some extent as shown in split-root and split-column experiments. However, a young, defense-wise unprepared plant placed in the same spot may not be able to cope with this situation and thus show severe symptoms, which is characteristic for ARD.

8 References

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Peer reviewed publications

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Conference contributions

Oral presentations

Rohr, A.-D., Reim, S., Weiß, S., Liu, B., Beerhues, L., Schmitz, M., Hanke, M.-V., Winkelmann, T., Flachowsky, H.: Identification of early markers for apple replant disease (ARD) at transcriptomic level. 53. Gartenbauwissenschaftliche Jahrestagung der DGG und des BHGL 2019, Berlin 06.-09.03.2019

Rohr, A.-D., Liu, B., Beerhues, L., Winkelmann, T.: Comparison of early reactions of apple rootstock M26 growing in soil from three apple replant disease sites in regard of growth, candidate gene expression and phytoalexin content. GRK1798 “Signaling at the Plant - Soil Interface” Doctoral Researchers’ Conference 2017, Bad Salzdetfurth 27-29.09.2017

Poster presentations

Reim, S., Rohr, A.-D., Winkelmann, T., Cestaro, A., Flachowsky, H.: Identification of genetic factors influencing replant disease in apple (ARD). BonaRes Statusseminar 2020, Leipzig 17.-19.02.2020

Liu, B., Rohr, A.-D., Mahnkopp-Dirks, F., Weiß, S., Winkelmann, T., Beerhues, L.: Phytoalexin production and exudation by roots of *Malus* genotypes grown in ARD soils. BonaRes Statusseminar 2019, Leipzig 19. - 21.02.2019

Rohr, A.-D., Liu, B., Beerhues, L., Winkelmann, T.: Early reactions of apple rootstock ‘M26’ growing in three apple replant disease soils in terms of growth, candidate gene expression and phytoalexin content. BONARES 2018 Conference – Soil as Sustainable Resource, Berlin 26.-28.02.2018

Rohr, A.-D., Bündig, C., Bartsch, M., Winkelmann, T.: Expression analysis of selected genes in starch potato (*Solanum tuberosum* L.) subjected to osmotic stress in vitro. 5. Quedlinburger Pflanzenzüchtungstage in Kombination mit der Jahrestagung der GPZ AG Genomanalyse inkl. 18. Kurt von Rümker Vorträge, Gatersleben 1.-7.3.2017

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