Transcriptomic analysis of molecular responses in *Malus domestica* 'M26' plants affected by apple replant disease

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Abstract

Apple is one of the most important fruits cultivated all around the world with a high economic but also health beneficial value. Over the last 50 years, the high demand for apple and economical reasons resulted in high-density orchards and tree nurseries where plants are frequently replanted. The long-described worldwide phenomenon of apple replant disease (ARD) presents a significant difficulty in maintaining yield in centers of production nowadays, as chemicals and fumigants which were effective in controlling the disease are phased-out due to environmental concerns. ARD is thought to be caused by detrimental microorganisms accumulating in the rhizosphere, mainly representing fungi and oomycetes of the genera *Rhizoctonia* spp., *Cylindrocarpon* spp., *Pythium* spp. and *Phytophthora* spp. but recently also allelochemicals have been reported to play an important role. Nevertheless, it can be concluded that complex interactions between both biotic and abiotic factors lead to ARD. Affected plants are diminished in their plant growth and yield, and they demonstrate delayed fruit production. However, molecular reactions in planta are not well understood yet, only few studies have focused on this aspect. To unravel disease etiology this study aimed to uncover transcriptomic responses of the ARD susceptible apple rootstock 'M26' on different ARD soils and at several time points.

In 2010, the genome sequence of the apple cultivar 'Golden Delicious' was published. A new technology in RNA sequencing, namely the massive analysis of cDNA ends (MACE) was employed to accomplish the goal of this study. MACE sequencing data was complemented by RT-qPCR experiments further supporting gene expression results. Gene expression was analyzed in the whole root system and the three youngest fully developed leaves of plants cultivated in ARD and γ-irradiated ARD soils differing in disease severity. Next to shoot and root growth depression, typical biotic stress response genes involved in plant defense were observed to be differentially regulated in ARD affected plants, also in a time-dependent pattern. This led to the conclusion that observed typical defense reactions towards biotic stress were expressed, but not effective in 'M26'.

Genes in secondary metabolite production as well as plant defense, regulatory and signaling genes were upregulated in ARD roots, whereas for several genes involved in primary metabolism lower expression was detected. Examined genes exhibited mostly conserved expression in 'M26' roots cultivated on different ARD soils. Most interestingly, genes involved in phytoalexin (PA) biosynthesis were consistently upregulated in ARD roots. In leaves of ARD challenged plants, different genes were affected and in particular, there were hints at the occurrence of potential systemic oxidative stress. A delayed systemic response on the transcriptomic level might be deduced from the increase in many PA genes starting at days 10-14 whereas this reaction to ARD was already observed after 3-7 days in most cases in roots.

PAs present a specialized form of defense mainly against fungi and the PA biosynthesis genes biphenyl synthase, *O*-methyltransferase and biphenyl-4-hydroxylase were consistently upregulated in ARD variants. The strong and early expression of these genes correlated with the very high concentrations of actual products in root material, namely 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzofuran, 2'-hydroxyaucuparin and noreriobofuran. It was the first time that these PAs were detected, identified and quantified in apple roots. In particular, 3-hydroxy-5-methoxybiphenyl and aucuparin were exclusively found in ARD samples. Their accumulation up to 1.9 mM led to the assumption that the impaired sequestration and/or exudation of the potentially cytotoxic PAs and oxidative stress may lead to root damage in ARD soils, but regarding the high PA contents, further tests have to be conducted.

Key words: Apple replant disease, phytoalexins, transcriptomic analysis

Zusammenfassung

Der Apfel ist mit seinen hohen ökonomischen, aber auch gesundheitsfördernden Eigenschaften eine der wichtigsten Früchte, und wird weltweit angebaut. In den letzten 50 Jahren führten die hohe Apfelnachfrage und ökonomische Gründe zu dichten und häufig neu bepflanzten Beständen in Obstplantagen und Baumschulen. Das altbekannte, weltweite Phänomen der Apfel-Nachbaukrankheit (ARD) stellt heutzutage ein großes Problem dar, gewünschte Erträge in Produktionszentren zu erreichen, da die Nutzung bislang eingesetzter Chemikalien und Entseuchungsmittel aufgrund von Umweltbedenken eingeschränkt wurde. ARD wird wahrscheinlich durch schädliche Mikroorganismen in der Rhizosphäre verursacht, wobei vor allem Pilze und Oomyceten der Gattungen *Rhizoctonia* spp., *Cylindrocarpon* spp., *Pythium* spp. und *Phytophthora* spp. eine wichtige Rolle spielen, aber vor kurzem wurde auch Allelochemikalien eine wichtige Rolle attestiert. Letztendlich führen komplexe Wechselwirkungen zwischen biotischen und abiotischen Faktoren zu ARD. Betroffene Pflanzen werden in ihrem Wachstum und ihrem Ertrag gemindert und zeigen eine verzögerte Fruchtentwicklung. Allerdings sind molekulare Reaktionen in planta noch nicht gut erforscht. Um den Krankheitsverlauf zu entschlüsseln, zielte diese Studie darauf ab, transkriptomische Reaktionen der ARD-empfindlichen Apfelunterlage ‘M26’ auf verschiedenen ARD-Böden und zu verschiedenen Zeitpunkten aufzudecken.

Im Jahr 2010 wurde die Genomsequenz der Apfelsorte ‘Golden Delicious’ veröffentlicht. Um das Ziel dieser Studie zu erreichen, wurde die neue RNA-Sequenzierungstechnologie ‘massive analysis of cDNA ends’ (MACE) eingesetzt. Genexpressionsergebnisse der MACE-Sequenzierungsdaten wurden durch RT-qPCR Experimente unterstützt. Die Genexpression wurde im gesamten Wurzelsystem und den drei jüngsten vollständig entwickelten Blättern von Pflanzen untersucht, die in ARD- und γ-bestrahlten ARD-Böden kultiviert wurden. Neben der Spross- und Wurzelwachstumsdepression wurde die differentielle, zeitabhängige Regulation biotischer Stressreaktions-Gene in ARD-Pflanzen beobachtet. Dies führte zu der Schlussfolgerung, dass Verteidigungsreaktionen gegen biotischen Stress stattfanden, aber nicht wirksam in ‘M26’ waren.

Gene in der Sekundärmetabolitproduktion und Signaltransduktion sowie Pflanzenverteidigungs- und Regulierungsgene wurden in ARD-Wurzeln hochreguliert, während für mehrere Gene, die am primären Metabolismus beteiligt waren, eine geringere Expression nachgewiesen wurde. Die untersuchten Gene wurden auf verschiedenen ARD-Böden in ‘M26’-Wurzeln konserviert exprimiert. Interessanterweise wurden Phytoalexin (PA) Biosynthese Gene in ARD-Wurzeln konstant hochreguliert. In Blättern von ARD-Pflanzen waren andere Gene betroffen, insbesondere gab es Hinweise auf das Auftreten von potentiell systemischem oxidativen Stress. Der Anstieg vieler PA-Gene ab Tag 10-14 könnte als verzögerte systemische Reaktion auf transkriptomischer Ebene gedeutet werden, während ARD-Wurzeln in den meisten Fällen bereits nach 3-7 Tagen diese Reaktion zeigten.

In ARD-Varianten wurden Biphenyl-Synthasen, *O*-Methyltransferasen und Biphenyl-4-Hydroxylasen konstant hochreguliert. Im Wurzelmaterial korrelierte ihre Expression mit sehr hohen Konzentrationen an 3-Hydroxy-5-Methoxybiphenyl, Aucuparin, Noraucuparin, 2-Hydroxy-4-Methoxydibenzofuran, 2'-Hydroxyaucuparin und Norerioboфuran. Zum ersten Mal wurden diese PAs in Apfelwurzeln detektiert, identifiziert und quantifiziert. 3-Hydroxy-5-Methoxybiphenyl und Aucuparin wurden ausschließlich in ARD-Proben gefunden. Konzentrationen bis zu 1.9 mM führten zu der Annahme, dass die beeinträchtigte Sequestrierung und/oder Exsudation der potentiell zytotoxischen PAs und oxidativer Stress zu Wurzelschäden in ARD-Böden führen könnte, aber in Bezug auf den hohen PA-Gehalt müssen weitere Tests durchgeführt werden.

Schlagwörter: Apfel-Nachbaukrankheit, Phytoalexine, Transkriptomanalyse

Contents

Abbreviations

γARD	γ-irradiated apple replant disease
ABA	abscisic acid
ABC	ATP-binding cassette
ANOSIM	analysis of similarities
ARD	apple replant disease
ATP	adenosine triphosphate
B4H	biphenyl-4-hydroxlase
BAP	benzylaminopurine
BIS	biphenyl synthase
bHLH	basic/helix-loop-helix
cDNA	complementary DNA
CoA	coenzyme A
CYP	cytochrome P450 dependent hydroxylase
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
ET	ethylene
ETI	effector-triggered immunity
FDR	false discovery rate
GST	glutathione S-transferase
HR	hypersensitive response
HSP	heat shock protein
IBA	indole-3-butyric acid
JA	jasmonic acid
MACE	massive analysis of cDNA ends
MAMP/PAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NBS-LRR	nucleotide binding-site-leucine-rich repeat
NO	nitric oxide
OMT	*O*-methyltransferase
PA	phytoalexin
PCA	principal component analysis
PR	pathogenesis-related
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
RLK	receptor-like kinase
RLP	receptor-like protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SA	salicylic acid
UDP	uridine diphosphate
UGT	UDP-glucosyltransferase

1. General introduction

1.1. The worldwide importance of apple

Apple (*Malus domestica*) belongs to the Rosaceae family which includes other commercial fruits such as apricot, cherry, peach, pear and strawberry but also ornamental plants like rose (Park et al. 2006). The fruit tree is produced throughout the temperate zone but recently also in subtropical and tropical zones (Velasco et al. 2010; Brown 2012). Worldwide, apple trails only bananas in total production quantity with 84,630,275 t versus 114,130,151 t and was harvested on a total area of 5,051,851 ha following grapes (7,124,512 ha, 74,499,859 t) and bananas (5,393,811 ha) in 2014 (FAOSTAT 2014). By far, Asia produces the most apples with 62.7 % followed by Europe with 20.7 % (FAOSTAT 2014). In particular, China can be regarded as the main producer of apples, followed by the USA where apple is the third most valuable fruit crop after grapes and oranges (Brown 2012). Economically, in 2006, apple production was worth approximately $1.6 billion annually in the USA (Park et al. 2006) and has increased to more than $2.5 billion dollars annually in 2012 (Brown 2012). The reason for the high demand of apples next to its pleasant taste is their potential effect on improved health in humans due to its phytochemical profile reducing risks of chronic diseases, exhibiting antioxidant, antiproliferative and cell signaling effects (Hyson 2011).

The high demand for apple led to an increase of production over the last 50 years which was achieved by establishing size-controlling rootstocks for improving orchard practices to maximize precocity, quality and yield, therefore, leading to high-density orchards with higher economical value (Zhu et al 2001; St. Laurent et al. 2010; Byrne 2012). Nowadays, fruit producing areas propagate plants by grafting buds of a scion onto rootstocks which are propagated from rooted vegetative cuttings in nursery layering beds (Gessler and Patocchi 2007; St. Laurent et al. 2010; Volk et al. 2015). Trees can remain in production for 30 or more years, but may be replanted on average every 12-16 years in high density orchards (Volk et al. 2015). Today, commercial orchards are uniform which is covetable agronomically but it is fatal for resistance toward pests and ensuing diseases (Gessler and Patocchi 2007; Mazzola et al. 2009).

1.2. Apple genetics

The domesticated apple is thought to be an offspring of *M. sieversii* which hybridized with European and Asian species (Velasco et al. 2010; Brown 2012). In general, genetic diversity in apple is high (Isutsa and Merwin 2000; Gessler and Patocchi 2007; Mazzola et al. 2009). The genus comprises a variety of species which are closely related and easily crossable (Gessler and Patocchi 2007). Although the highly heterozygous apple is an allopolyploid, it generally behaves like a diploid due to sufficient diversity of the parental chromosomes with a haploid chromosome number of $x = 17$ (Velasco et al. 2010; Brown 2012) but it can also occur as tri- and tetraploids, with higher ploidy levels concurring with increased sizes of pollen grains, stomata, flowers, fruits and leaves but decreased pollen viability (Podwyszyńska et al. 2016). However, it displays self-incompatibility

leading to high heterozygosity (Gessler and Patocchi 2007) and requires cross-pollination (Byrne 2012). In 2010, the genome sequence of the diploid apple cultivar 'Golden Delicious' was published (Velasco et al. 2010). This revealed a probable genome duplication in an ancestral 9-chromosome genome event 50 million years ago resulting in a genome with 57,386 predicted genes (Velasco et al. 2010). The 17-chromosome karyotype was the consequence of interchromosomal reorganizations and the ensuing loss of a single chromosome but it was suggested that the specific fruit type evolved after the genome-wide duplication event (Velasco et al. 2010).

1.3. Definition of apple replant disease

Mazzola and Manici (2012) presented several terms for the observed diminished crop productivity at production centers with intensive replanting practices. They listed soil sickness, soil exhaustion, replant disorder, replant problem and replant disease as synonyms for weak tree growth and reduced yields over time probably caused by detrimental soilborne microorganisms. Their definition of replant disease, which focuses on biotic factors being responsible for the disease, will be used in this study.

Plant species that are affected by replant disease include apple, peach, citrus, grapes, cherries, strawberry, black walnut and roses (Gur and Cohen 1989; Singh et al. 1999; Zhang et al. 2007; Manici and Caputo 2010). Apple replant disease (ARD) in particular affects orchards of apple and is defined as a consequence of replanting apple trees in the same field due to e.g., renewal because of age, or rootstock production in tree nurseries leading to slow and diminished shoot growth shortening orchard life (Mazzola and Manici 2012). Hereby, replanting after 1-2 years can already lead to plant growth differences (Mazzola 1999). Although replant disease has been documented for more than 200 years (Mai and Abawi 1981), the complex phenomenon has not been unraveled yet. Astonishingly, replant disease persists in soils even after plants have been removed a long time ago (Mazzola and Mullinix 2005; Van Schoor et al. 2009; Tewoldemedhin et al. 2011c).

1.4. ARD symptoms

ARD is an important problem in the production of apple worldwide in all of the major fruit-growing regions resulting in huge economic losses (Mazzola 1998; Yin et al. 2016). Profitability is reduced by 50 % throughout the life span of the orchard due to delayed production caused by ARD as affected trees start bearing fruits 2-3 years later than unaffected trees and fail to yield amounts comparable to their healthy counterparts (Mazzola 1998; Van Schoor et al. 2009). ARD affects young trees, results in uneven growth and under severe disease pressure even death of trees can be observed (Mai and Abawi 1981; Mazzola 1998).

Symptoms are observed under field conditions shortly after planting within 1-3 months as ARD affected plants are characterized by drastic stunting and shortened internodes as well as rosetted leaves aboveground (Caruso et al. 1989; Mazzola 1998; Mazzola and Manici 2012). Under greenhouse conditions symptoms can already be visualized within two to five weeks (Yim et al. 2013, 2015). Belowground, ARD challenged plants exhibit general reduction in root biomass as well as small root

systems and decayed in addition to discolored roots, and it was reported that epidermal cells plus cortical tissues are prematurely destroyed resulting in reduction of lateral root expansion and fewer functional root hairs (Jaffee et al. 1982; Caruso et al. 1989; Mazzola 1998; Mazzola and Manici 2012; Yim et al. 2013; Atucha et al. 2014; Shin et al. 2014; Henfrey et al. 2015). Eventually, productivity of these plants is greatly diminished due to decrease in overall fruit yield and quality demonstrated by undesirable texture, appearance and flavor of apple fruits (Mazzola 1998; Mazzola and Manici 2012; Liu et al. 2014). In several studies it was shown that the apple rootstock 'M26' can be used as a reliable indicator test plant to detect ARD in soils due to its high susceptibility towards the problem (St. Laurent et al. 2010; Yim et al. 2013, 2015).

1.5. Abiotic and biotic factors causing ARD

Potential causes inciting ARD differed between geographic regions or between orchards in the same region. Abiotic factors involving soil pH, structure and drainage, nutritional status, heavy metal contamination, as well as cold or drought stress may contribute to plant growth inhibition in ARD (Mai and Abawi 1981; Willett et al. 1994). Soil extracts and microbial toxins reduced plant growth (Gur and Cohen 1989; Tagliavini and Marangoni 1992), furthermore, autotoxicity, a form of intraspecific allelopathy, of apples was found in ARD where the release of toxic chemicals into the soil inhibited plant growth (Singh et al. 1999). Moreover, in ARD the role of root exudates was discussed (Börner 1959; Wittenmayer and Szabó 2000; Hofmann et al. 2009) and also in the citrus replant disease, compounds were extracted causing severe growth reduction hinting at an important role of allelopathy in replant disease (Burger and Small 1983; Hassan et al. 1989a, b). Recently, more studies have been conducted in analyzing the role of allelochemicals and root exudates in ARD (Manici et al. 2016; Yin et al. 2016, 2017). But Mazzola and Manici (2012) remarked that due to the persistence of replant disease over a number of years (Mazzola and Mullinix 2005; Van Schoor et al. 2009; Tewoldemedhin et al. 2011c), toxins would have to exhibit very high stability and resiliency to microbial degradation.

As soil fumigation or pasteurization is able to majorly improve apple plant growth (Mai and Abawi 1981; Jaffee et al. 1982; Mazzola 1998; Isutsa and Merwin 2000), it was concluded that the disease was primarily caused by biological stressors rather than it was the result of abiotic factors, but potential causal agents of ARD can also differ between different regions or even orchards of the same region (Mazzola 1998; Manici et al. 2003). Actinomycetes were found in ARD soils but they did not show any pathogenicity towards apple plants (Tewoldemedhin et al. 2011c) whereas Acidobacteria were found to be negatively correlated with plant growth in apple (Nicola et al. 2017). However, Mazzola (1998) was able to show that bacteria did not play a major role in ARD etiology as elimination of bacteria with chloramphenicol did not improve plant growth. In fact, elimination of fungi improved apple plant growth and it was concluded that fungi play the dominant role in ARD soils worldwide (Mazzola 1998).

Only a minor role was attributed to *Fusarium* spp. as solitary *F. solani* revealed low pathogenicity towards apple plants (Manici et al. 2003; Van Schoor et al. 2009; Tewoldemedhin et al. 2011b). However, in ARD soils coming from locations all over the world, pathogenicity was found for *Rhizoctonia* spp. with *R. solani* being most abundant (Jaffee et al. 1982; Mazzola 1998, 1999; Manici et al. 2003, 2015; Van Schoor et al. 2009; Tewoldemedhin et al. 2011b). In addition, worldwide, *Cylindrocarpon* spp. was consistently found in ARD soils and revealed pathogenicity towards plants (Jaffee et al. 1982; Mazzola 1998; Manici et al. 2003, 2013, 2015; Van Schoor et al. 2009; Tewoldemedhin et al. 2011a, c; Franke-Whittle et al. 2015). The oomycetes *Pythium* spp. (Mazzola 1998, 1999; Manici et al. 2003, 2013; Van Schoor et al. 2009; Tewoldemedhin et al. 2011b, c) and *Phytophthora* spp. (Mazzola 1998, 1999; Tewoldemedhin et al. 2011b, c) were constantly found in ARD soils all over the world as well. *Pythium* spp. and *Cylindrocarpon* spp. even showed a synergistic interaction effect on apple growth inhibition highlighting complex interactions responsible for causing ARD (Tewoldemedhin et al. 2011c). Although the importance of fungi and oomycetes in the disease etiology of ARD was evident, the relative contribution of the different species of the presented genera and also the comparative input of genera themselves differed among ARD soils of different locations (Jaffee et al. 1982; Mazzola et al. 1998; Manici et al. 2003; Van Schoor et al. 2009; Manici and Caputo 2010; Tewoldemedhin et al. 2011a, b).

Additionally, *Pratylenchus* spp. especially *P. penetrans* was found in ARD soils but they were deemed to play, at the most, only a minor role in ARD etiology (Jaffee et al. 1982; Mazzola 1998; Van Schoor et al. 2009). Interestingly, healthy apple plants were more often colonized by vesicular-arbuscular mycorrhizal fungi possibly due to few functional root hairs presenting less penetration sites for an infection in ARD affected plants and, therefore it was suggested that lack of endomycorrhizae was associated with ARD (Caruso et al. 1989).

1.6. ARD counteractions

Counteractions to effectively alleviate or surmount ARD are of utmost importance in the sustainable development of apple production (Tewoldemedhin et al. 2011b). In former times, the control of ARD was mainly achieved by biologically broad-spectrum soil fumigants such as methyl bromide or chloropicrin as they provided effective control (Covey et al. 1979; Mai and Abawi 1981; Gur et al. 1991; Willet et al. 1994; Mazzola and Gu 2002). However, they represent more than a few problems including application intricacy, high cost as well as hazards to the environment and human health. Furthermore, their efficacy greatly relies on soil moisture and temperature (Mazzola 1998; Mazzola and Manici 2012). For ethylene dibromide, very high persistence up to 19 years was found under field conditions and it could be a continuous source of groundwater contamination (Steinberg et al. 1987). In addition, it was reported that soil fumigation sometimes led to decreased plant growth itself possibly due to direct toxicity of remaining fumigant residues or elimination of beneficial microorganisms like mycorrhizal fungi (Mai and Abawi 1981).

Alternative counteractions are the focus of newer research as preplant soil fumigation does not constantly prevent ARD at all sites and the phase-out of widely used preplant fumigants instills a sense of urgency (Mazzola et al. 2002; Porter et al. 2010). Compost application had an effect on soil microbial communities but did not improve plant growth in New York, USA (Yao et al. 2006) whereas better plant growth under ARD conditions was observed in South Africa (Van Schoor et al. 2009). But in South Tyrol, organic soil amendments even led to an enhancement in *Rhizoctonia* spp. and *Pythium* spp. due to increases in organic carbon as a result of decaying crop debris (Manici et al. 2003). Mazzola et al. (2001) stated that negative side effects may be avoided by highly degraded or humified composts. These divergent results indicate that compost application as an optional method to avoid ARD is highly variable in its effectiveness.

Another approach was based on the fact that certain pathogenic soil microorganisms may be suppressed via selective microbial community shifts as the introduction or enhancement of biological antagonists to ARD inciting microorganisms was reported to induce soil suppressiveness and it could be shown that *Burkholderia cepacia* and *Pseudomonas putida* were important in suppressing ARD due to antagonistic effects towards *R. solani* and *Pythium* spp. (Mazzola 1999; Mazzola et al. 2002; Gu and Mazzola 2003; Manici et al. 2015). Additionally, biofumigation via incorporation of Brassicaceae plants or Brassicaceae seed meal containing high amounts of glucosinolates into soil was able to suppress *Rhizoctonia* spp., *Cylindrocarpon* spp. as well as *P. penetrans* and improved apple plant growth in ARD soil (Mazzola et al. 2001; Mazzola et al. 2002; Yim et al. 2016). However consistent results were not achieved at different sites of ARD occurrence (Mazzola and Mullinix 2005; Mazzola and Manici 2012; Mazzola et al. 2015). Furthermore, the method results in lost production of orchards due to time-consuming generation of green manure crops (Mazzola and Manici 2012) and in some cases phytotoxicity occurred even after the recommended delay of planting (Mazzola et al. 2001). Hence, soil and site dependent differences make biofumigation an unreliable method as external factors including temperature, precipitation and solar radiation as well as tissue disruption and soil water content influence the efficacy (Yim et al. 2016).

Due to the economic importance of apple, additional alternative ARD counteractions have been studied ranging from treatments for manipulation of rhizosphere microbial communities to decrease detrimental and increase beneficial microorganisms (Manici et al. 2013, 2015; Caputo et al. 2015; Yim et al. 2015), over carbon source-dependent anaerobic soil disinfestation (Hewavitharana et al. 2014; Hewavitharana and Mazzola 2016) to the application of biochar (Wang et al. 2014) as well as intact glucosinolates (Hanschen et al. 2015), and arbuscular mycorrhiza or microbial (Guo et al. 2014; Gastol and Domagala-Swiatkiewicz 2015) as well as seaweed fertilizers (Wang et al. 2016).

Furthermore, genotypic differences and orchard replant position were deemed more important to influence tree growth and microbial communities than fumigation in ARD (Rumberger et al. 2004). In previous tree rows, plants were severely affected and rhizosphere bacterial composition differed significantly compared to plants of inter-row grass lanes (Mazzola 1999; Rumberger et al. 2004).

Therefore, another counteraction to reduce ARD was suggested to be avoiding the direct replanting in tree holes of preceding plants (Granatstein and Mazzola 2001; Rumberger et al. 2004; Leinfelder and Merwin 2006; Kelderer et al. 2012; Yin et al. 2016). In addition, soil microbial community composition and the severity of ARD were influenced by rootstock genotype succession, and ARD tolerance was attributed to a greater abundance of antagonistic bacteria in the rhizosphere (St. Laurent et al. 2010), and in general it was remarked that host genetics is able to impact soil microbial communities in bulk and rhizosphere soil leading to disease control mechanisms (Mazzola and Gu 2000; Mazzola and Zhao 2010; Mazzola and Manici 2012). Likewise, lesion nematode populations were tolerated more by rootstocks of the Geneva series which then exhibited less susceptibility to root infection by *Pythium* spp. than rootstocks from the Malling series (Mazzola et al. 2009).

The release of the genome sequence of apple already hinted at a large number of potential resistance genes available (Velasco et al. 2010). It could be shown that some apple rootstocks of the Cornell-Geneva rootstock breeding program which emphasized selection for genotypes with multiple disease resistance were more tolerant towards the ARD complex and employing more tolerant rootstocks might be a suitable counteraction (Isutsa and Merwin 2000; Leinfelder and Merwin 2006; Yao et al. 2006; Mazzola et al. 2009; St. Laurent et al. 2010). Rootstock breeding (Volk et al. 2015) plus evaluation of ARD tolerance have recently been described (Forge et al. 2016). Furthermore, phenotypical information referring to root turnover (Atucha et al. 2014) as well as root development (Emmett et al. 2014) have been linked to ARD tolerance mechanisms.

The use of up to date DNA-based breeding methods including genetic maps, identification of trait associated markers and marker-assisted breeding can help in breeding improved cultivars (Zhu et al. 2001; Gessler and Patocchi 2007). However, molecular reactions in planta have to be better understood to unravel disease etiology for the development of ARD trait associated markers (Zhu et al. 2014). Data so far is scarce, but it could be shown that plants exposed to ARD reacted with higher phenolic compound contents (Henfrey et al. 2015), probably involving phloridzin being exuded into the soil (Hofmann et al. 2009; Emmett et al. 2014; Yin et al. 2016, 2017). More recently, Shin et al. (2014, 2016) uncovered gene expression patterns in apple roots after infection with *Pythium ultimum*, one of the many potential causal agents of ARD. Ethylene and jasmonate biosynthesis were upregulated in infected root tissue but also cytokinin biosynthesis and signaling were induced next to genes in secondary metabolite biosynthesis, cell wall fortification and plant defense (Shin et al. 2014, 2016; Zhu et al. 2014).

1.7. Biotic stress responses in general

Although the molecular biotic stress response of ARD challenged plants is not yet understood, in general, responses towards pathogens including fungi, bacteria and nematodes result in biotic stress and are diverse in nature (Dangl and Jones 2001). Plants possess the ability of passive protection against pathogens via preformed antimicrobial compounds called phytoanticipins (Van Etten et al.

1995), but specific defense responses rely on pathogen detection via microbe-associated molecular patterns (MAMP/PAMP, Boller and Felix 2009; De Coninck et al. 2014; Huot et al. 2014; Pandey et al. 2016). Membrane-bound pattern recognition receptors (PRRs) such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs) are responsible for detecting MAMP molecules, lead to PAMP-triggered immunity (PTI) and invoke further signaling molecules involved in PTI and other defense responses like calcium influx, alkalinization of the extracellular space, generation of nitric oxide (NO) and transcriptional reprogramming (Figure 1.1, Boller and Felix 2009; De Coninck et al. 2014; Huot et al. 2014; Pandey et al. 2016).

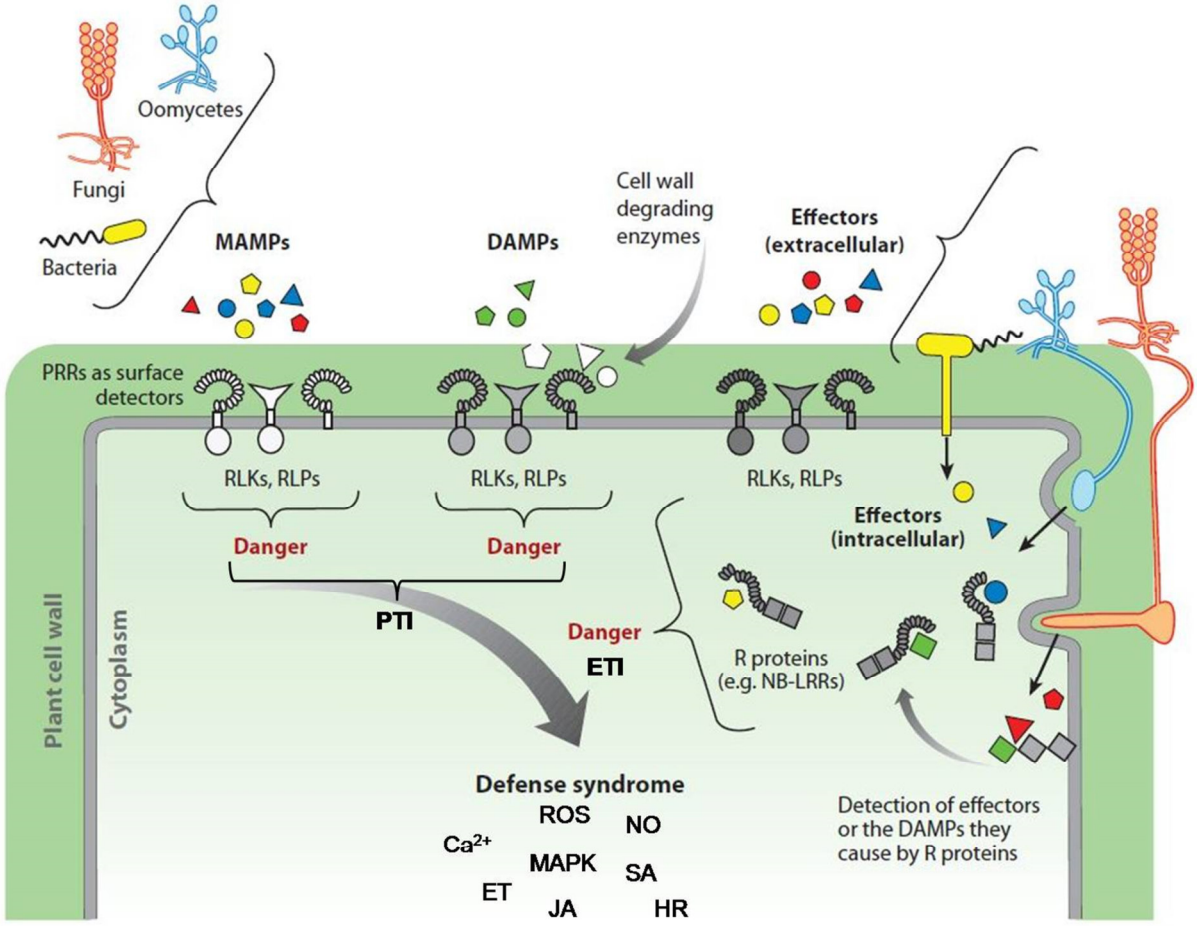

Figure 1.1 Overview of biotic stress response signaling according to Boller and Felix (2009, modified). Microbe-associated molecular patterns (MAMPs/PAMPS), damage-associated molecular patterns (DAMPs) and effectors are recognized as signals of danger by the plant leading to PAMP-triggered immunity (PTI) and/or effector-triggered immunity (ETI). Pattern recognition receptors (PRRs) such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs) can detect MAMPs and DAMPs. Effectors can be perceived by resistance (R) proteins with nucleotide binding-site-leucine-rich repeats (NB[S]-LRR). When MAMPs, DAMPs and effectors are recognized, a defense response (defense syndrome) is induced resulting in the generation of Ca^{2+}, reactive oxygen species (ROS), nitric oxide (NO) as well as mitogen-activated protein kinase (MAPK) signaling and ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) production ending, eventually, in hypersensitive responses (HR).

Similar to MAMPs, damage-associated molecular patterns (DAMPs) invoked by the damage caused by pathogens may occur from the plant itself, e.g. cell wall fragments, which are recognized by PRRs as well (Boller and Felix 2009). Mitogen-activated protein kinase (MAPK)-dependent signaling cascades, generation of reactive oxygen species (ROS) and transcription of defense-related genes are subsequently activated (Figure 1.1, Scheel 1998; Dangl and Jones 2001; Boller and Felix 2009; De Coninck et al. 2014; Zhu et al. 2014; Pandey et al. 2016). Especially, transcriptional reprogramming

can be observed quickly after infection through activation of defense genes via ROS, NO and MAPK signaling-induced activation of transcription factors (Durrant et al. 2000; De Coninck et al. 2014; Pandey et al. 2016). If pathogens are able to suppress this kind of defense via masking MAMPs with effectors, plants can counter with highly specific effector-triggered immunity (ETI) recognizing effectors via resistance gene products with nucleotide binding-site-leucine-rich repeats (NBS-LRR, Boller and Felix 2009; De Coninck et al. 2014; Pandey et al. 2016). Overall, plants perceive MAMPs, DAMPs and effectors all as signals of danger (Boller and Felix 2009) and resistance proteins exist both as cytoplasmic and transmembrane classes in multiple types so that both secreted ligands and surface components can be detected leading to the accumulation of inducible antimicrobial compounds (Dangl and Jones 2001). This often results in hypersensitive responses (HRs) e.g., cell death to prevent pathogen propagation and spread (Ding et al. 2007; Boller and Felix 2009; De Coninck et al. 2014; Huot et al. 2014). Furthermore, defense associated genes result in complex interaction signaling pathways leading to biosynthesis of ethylene (ET), jasmonic (JA) and salicylic acid (SA), cell wall fortification and lignification (Scheel 1998; Lam et al. 2001; Boller and Felix 2009; Lee and Lu 2011; De Coninck et al. 2014; Pandey et al. 2016).

1.8. Phytoalexin biosynthesis as a specialized biotic stress response

Major antimicrobial compounds produced against pathogen attacks are biosynthetically linked via the shikimic acid pathway (Dixon et al. 2001). Whereas phytoanticipins are formed as antimicrobial compounds under normal plant development, phytoalexin (PA) compounds are produced upon biotic stress to protect the plant from disease, mainly from fungal attacks (Van Etten et al. 1995; Dixon et al. 2001; Ahuja et al. 2012). PAs present a specialized form of defense, and in Rosaceae species two particular classes of PAs, namely biphenyls and dibenzofurans can be formed upon pathogen attack (Kokubun and Harborne 1995; Dixon 2001; Chizzali and Beerhues 2012; Khalil et al. 2013, 2015). In genera of this family 10 biphenyls and 17 dibenzofurans have been identified so far and elicitation was limited to members of this family only (Kokubun et al. 1995; Hüttner et al. 2010; Chizzali and Beerhues 2012). The simultaneous expression of these two classes of compounds with antibacterial and antifungal properties suggested a biosynthetic relationship directed at inhibiting growth of pathogens during plant-pathogen interactions (Hrazdina et al. 1997; Hüttner et al. 2010; Teotia et al. 2016). Biphenyls and dibenzofurans locally produced at infection sites acted in defense to inhibit spore germination, germ-tube development and mycelial growth (Kokubun et al. 1995; Hrazdina et al. 1997). Also in cytological studies on the PA camalexin and its antimicrobial activity in *Arabidopsis thaliana* in response to infection with *Botrytis cinerea* and *Alternaria* spp., PAs caused the disruption of membrane integrity (Rogers et al. 1996), inhibition of conidial germination and germ-tube elongation (Sellam et al. 2007) as well as the apoptotic-like programmed cell death of fungi (Shlezinger et al. 2011; Ahuja et al. 2012). Rogers et al. (1996) mentioned a majority of cases showing the correlation of host resistance and the level of PA accumulation in potato, *Vicia fabia* and

Phaseolus vulgaris. Likewise, in apple, fire-blight and scab tolerance was correlated to accumulation of biphenyls and dibenzofurans whereas susceptible genotypes failed to produce these PAs (Borejsza-Wysocki et al. 1999). However, it was also reported that *Brassica* spp. as well as various cruciferous plants did not show this typical correlation, and PA accumulation even resulted in cytotoxicity (Rogers et al. 1996).

Aucuparin and noraucuparin were the most abundant biphenyls found in Rosaceae species (Chizzali and Beerhues 2012). Regarding their biosynthesis, biphenyl synthase (BIS), a type III polyketide synthase, was found to be the key enzyme in providing the carbon skeleton for both biphenyls and dibenzofurans (Liu et al. 2004; Hüttner et al. 2010) which is expressed by a gene family in apple (Chizzali and Beerhues 2012). It was found to be induced after elicitation at the transcriptional level resulting in the enzyme catalyzing the iterative condensation of benzoyl-CoA with three malonyl-CoAs (Figure 1.2; Hüttner et al. 2010; Chizzali and Beerhues 2012). The linear tetraketide intermediate undergoes intramolecular aldol condensation and the loss of the terminal carboxyl group results in 3,5-dihydroxybiphenyl (Hüttner et al. 2010; Chizzali and Beerhues 2012). Response to fire-blight infection in apple trees resulted in differential regulation of four *BIS* genes and their PA products. It was observed that the BIS protein was localized in the junctions between neighboring cortical parenchyma cells suggesting an association of BIS with plasmodesmata (Chizzali et al. 2012a, b). Next to BIS, so far, also *O*-methyltransferases (OMTs, Khalil et al. 2015) and biphenyl-4-hydroxylases (B4Hs, Sircar et al. 2015) were identified as enzymes in PA biosynthesis for the production of the antifungal defense compounds in apple (Figure 1.2).

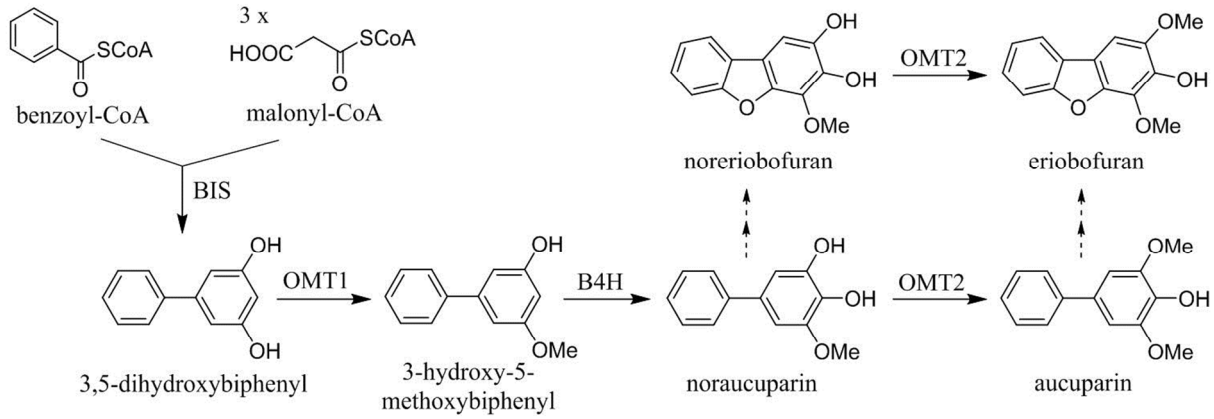

Figure 1.2 Proposed biosynthetic pathway of phytoalexins in apple according to Khalil et al. (2015, modified). Solid arrows represent identified enzymatic reactions whereas broken arrows mark unidentified enzymatic reactions. Biphenyl synthase (BIS) provides the carbon skeleton for both biphenyls and dibenzofurans. *O*-methyltransferases (OMT) and biphenyl-4-hydroxylases (B4H) modify compounds for the biosynthesis of the antifungal defense compounds in apple.

1.9. Thesis objectives

ARD represents an economic problem to apple producers around the world. Even though ARD has long been recognized and numerous studies have tried to uncover the disease etiology, no complete explanation has been found yet. As biotic stress is the main factor in causing ARD, identifying potential causal agents has been the research focus. But also the development of counteractions against ARD has been a major research question in previous studies. Nevertheless, studies have neglected the very important question of which molecular reactions are actually involved in ARD challenged plants.

As mentioned above, only recently, starting from 2014, a minority of studies has been conducted on molecular reactions in planta – this excludes earlier studies dealing with root exudates in ARD which only made assumptions on which molecular reactions are actually involved in the plant. Therefore, this study aimed to contribute to the elucidation of ARD etiology on a molecular level in the plant by analyzing transcriptomic responses of both roots – which are in direct contact with the biotic stressors – and leaves of the highly susceptible apple rootstock 'M26' to the presence or lack of biotic causal agents of ARD in different ARD soils. A transcriptomic analysis was performed, enabled by the availability of the apple genome sequence and it generates an overview of which genes are differentially expressed at what level. Massive analysis of cDNA ends (MACE) was employed as the method of choice because even low abundant transcripts can be effectively visualized at reduced costs compared to the more often used RNA sequencing analyses (Kahl et al. 2012). Specifically, the following questions – which will be presented in the subsequent chapters – were intended to be answered:

1. How do ARD affected 'M26' plants react to ARD on the transcriptomic level?

2. Does biotic stress by exposing the root system to ARD lead to a systemic response in aboveground tissue?

3. Are ARD affected molecular reactions in the plant conserved among different ARD soils?

4. Is there a time-dependent effect on ARD affected molecular reactions?

2. Transcriptomic analysis of molecular responses in *Malus domestica* 'M26' roots affected by apple replant disease

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Type of authorship	First author
Type of article	Research article
Contribution to the article	Performed the experiments, data analysis and wrote the manuscript
Co-authors contribution	Melanie Bartsch assisted in design of RT-qPCR experiments
	Traud Winkelmann conceived and coordinated the project, participated in interpretation of data and in writing the manuscript
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2.1. Manuscript

Abstract

Apple replant disease (ARD) leads to growth inhibition and fruit yield reduction in replanted populations and results in economic losses for tree nurseries and fruit producers. The etiology is not well understood on a molecular level and causal agents show a great diversity indicating no definitive cause, which applies to the majority of cases, has been found out yet. Hence, it is pivotal to gain a better understanding of the molecular and physiological reactions of the plant when affected by ARD and later to overcome the disease, for example by developing tolerant rootstocks.

For the first time, gene expression was investigated in roots of ARD affected plants employing massive analysis of cDNA ends (MACE) and RT-qPCR. In reaction to ARD, genes in secondary metabolite production as well as plant defense, regulatory and signaling genes were upregulated whereas for several genes involved in primary metabolism lower expression was detected. For internal verification of MACE data, candidate genes were tested via RT-qPCR and a strong positive correlation between both datasets was observed. Comparison of apple 'M26' roots cultivated in ARD soil or γ-irradiated ARD soil suggests that typical defense reactions towards biotic stress take place in ARD affected plants but they did not allow responding to the biotic stressors attack adequately, leading to the observed growth depressions in ARD variants.

Key words: Biotic stress response; gene expression; growth depression; MACE; plant defense; quantitative real-time PCR

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Introduction

The worldwide phenomenon apple replant disease (ARD) indicated by reduced shoot growth and fruit yields represents a severe problem to be overcome. Especially in tree nurseries growing Rosaceae species like apple this problem can be observed after replanting young trees (Yim et al. 2013). Symptoms can already be visualized directly after planting (Mazzola and Manici 2012) and include stunted growth, truncated internodes, rosetted leaves, small-sized root systems, rotten or discolored roots and reduced fruit yields (Mazzola 1998) as well as a loss of fruit quality (Mazzola and Manici 2012). Regarding the root system it can be observed that epidermal cells and cortical tissues are destroyed (Yim et al. 2013), root tip necrosis occur (Mazzola and Manici 2012), lateral root development is reduced (Savory 1966; Hoestra 1968) and functional root hairs are almost completely missing (Caruso et al. 1989). As a consequence, also the overall root biomass is generally reduced (Mazzola and Manici 2012). Apple is one of the most important cultivated tree fruits worldwide. In 2011, 75.5 million tons equaling about 12 % of the overall fruit production worldwide was credited to apples (FAOSTAT 2011) and apple production was estimated to account for 2.5 billion dollars per year in the USA alone (Brown 2012). Thus, it is essential to develop counteractions against ARD.

Although many studies focused on examining the causal agents of ARD, no definitive cause, which applies to the majority of cases, is identified up to now. Even though abiotic factors can hinder apple tree growth, ARD is associated to biotic causes as soil disinfection via pasteurization (Hoestra 1968; Jaffee et al. 1982a) or fumigation (Mai and Abawi 1981; Slykhuis and Li 1985) restores plant growth on replant soils. Jaffee et al. (1982a, b) and Mazzola (1998) reported on the causal agents potentially responsible for ARD. The authors mentioned fungal genera such as *Cylindrocarpon* (Braun 1991, 1995) and *Rhizoctonia* (Mazzola 1997), oomycete genera like *Phytophthora* (Sutton et al. 1981) and *Pythium* (Mazzola 1998) as well as the lesion nematode *Pratylenchus penetrans* (Gilles 1974; Mai and Abawi 1981). Especially, *Pythium ultimum* was reported several times in literature to be found in ARD affected soil (Mazzola 1997; Tewoldemedhin et al. 2011a, b) and recent studies dealing with gene expression started to uncover the response of roots towards infection with *P. ultimum* (Shin et al. 2014, 2016). However, the diversity in microorganisms that are potentially linked to ARD makes it difficult to effectively control the problem as broad-spectrum biocides have to be used, most of which are outbound in many parts of the world (Mazzola 1998). Porter et al. (2010) reported concerns for growers due to the phase out of soil fumigants which were successfully used for soil disinfection in former times. Yim et al. (2016) mentioned the EU directive EC 128/2009 (2009) as a goal for the EU to achieve a reduced and sustainable employment of pesticides, and recent studies investigating biofumigation as an alternative mean to disinfect ARD affected soil support this view (Mazzola et al. 2015). However, results still vary due to external factors like differences in temperature, precipitation and solar radiation as well as tissue disruption and soil water content playing a role in the effect of these alternative methods (Yim et al. 2016).

Only recently, studies started to focus on unraveling what actually happens in planta to examine affected molecular and physiological mechanisms involved in ARD. Higher contents of phenolic compounds were recorded in plants exposed to ARD due to the accumulation of antioxidant substances (Henfrey et al. 2015). Moreover, the flavonoid phloridzin was found to a higher extent in root exudates of ARD plants (Hofmann et al. 2009). Furthermore, infecting plants with one of the causal agents of ARD, *P. ultimum*, led to the upregulation of ethylene and jasmonate biosynthesis genes in root tissue (Shin et al. 2014). Recently, using an RNA-sequencing approach, Shin et al. (2016) presented a detailed picture of transcriptomic changes in apple roots in reaction to *P. ultimum* inoculation. In addition to ethylene and jasmonate, also cytokinin biosynthesis and signaling were found to be induced in the inoculated roots. Moreover, several genes involved in secondary metabolite biosynthesis, cell wall fortification and plant defense were upregulated in roots exposed to the pathogen.

Phytoalexins, another class of secondary metabolites, can have an impact on the defense response towards biotic stress as shown by their accumulation in the transition zone of *Erwinia amylovora* infected apple shoots (Chizzali et al. 2012). In phytoalexin biosynthesis, biphenyl synthases (Chizzali et al. 2012), *O*-methyltransferases (Khalil et al. 2015) and biphenyl-4-hydroxylases (Sircar et al. 2015) are responsible for the production of the antifungal defense compounds in the form of biphenyls and dibenzofurans. Both of these compound classes were already detected in Rosaceae species belonging to 14 genera including *Malus*, *Pyrus* and *Sorbus* (Chizzali and Beerhues 2012; Khalil et al. 2013, 2015). Chizzali and Beerhues (2012) reported ten different biphenyls and 17 dibenzofurans characterized in these plant species.

In biotic stress response, regulatory genes play an important role to maintain a balanced metabolism or, regarding transcription factors, can regulate specific genes or invoke signals in plants (Davletova et al. 2005; Li et al. 2014; Nuruzzaman et al. 2013; Yanagisawa 2002). It was shown that biotic stress triggers internal defense reactions of affected plants by activating signal transduction pathways (Dodds and Rathjen 2010; Tsuda and Katagiri 2010; Sato et al. 2010) in which ethylene (Broekaert et al. 2006; Ruduś et al. 2013) and gibberellin (De Bruyne et al. 2014) but also kinases (Afzal et al. 2008) can be involved.

This study aimed to better understand ARD effects on a molecular level in planta by analyzing transcriptomic responses of affected roots. These insights will enable the identification of markers for early reactions to ARD which might be used in the development of tolerant rootstocks for instance. As a test object, the apple rootstock ‘M26’ was subjected to soil affected by ARD or disinfected ARD soil. Recently, Yim et al. (2013) showed that ‘M26’ could be used as a reliable indicator test plant to detect ARD in soils due to its high susceptibility towards the problem. Employing the rather new RNA sequencing method “massive analysis of cDNA ends” – MACE (Kahl et al. 2012) – the fully sequenced genome of apple (Velasco et al. 2010) was used to unravel molecular reactions involved in ARD. MACE was applied, because the complexity of RNA samples is reduced by only 3’-end

sequencing and it allows the high resolution quantification of low expressed genes as well as the possibility for generation of gene specific markers (Kahl et al. 2012). The objective of this study was to analyze the transcriptomic response of apple rootstock 'M26' roots to the presence or lack of biotic causal agents of ARD in soil. A selection of candidate genes which was based on a putative function in biotic stress response was further studied in RT-qPCR experiments.

Material and methods

Soil origin and disinfection

In March 2014, soil for the biotest was obtained from a tree nursery in the area of Pinneberg, Schleswig-Holstein, Germany (53° 42' 18.81'' N, 9° 48' 16.74'' E). The soil was taken at a depth of 0-25 cm from three field plots. Until 2009 apple rootstock plants were grown on site, followed by *Prunus domestica* in 2010, *Cydonia oblonga* in 2011 and the apple rootstock 'M4' from 2012 (Yim et al. 2015, 2016). After homogenizing, half of the soil volume was sent for disinfection via γ-irradiation (BGS – Beta Gamma Service, Wiehl, Germany) at a minimum dose of 10 kGy by which actinomycetes, fungi and invertebrates are eliminated (McNamara et al. 2003). The soil was packed in autoclavable bags (Sarstedt, Nümbrecht, Germany) at a soil volume of 18-20 L and, after γ-irradiation, stored at 4 °C until one day before the start of the experiment (total storage duration: 10 days).

Plant cultivation

Plantlets of the highly susceptible apple rootstock 'M26' (Kviklys et al. 2008; St. Laurent et al. 2010; Yim et al. 2013, 2015) were propagated and rooted in vitro according to Yim et al. (2013) with the exception of using ½ MS basal medium with 2 % sucrose and 4.92 µM IBA (indole-3-butyric acid) without BAP (benzylaminopurine) for rooting. Afterwards rooted plants were acclimatized and in May 2014, four weeks after transfer to the greenhouse, potted into 1 L pots containing either γ-irradiated replant soil (γARD) or replant soil (ARD), each supplied with 2 g L^{-1} of the slow release fertilizer Osmocote Exact Standard 3-4 M (16-9-12+2MgO+TE, http://www.scottsprofessional.com). For the transcriptomic analysis, 25 replicates (= individual plants) per variant were used in addition to 5 replicates for recording the shoot length over the course of the experiment (Supplementary Fig. S1). Conditions in the greenhouse were as follows: 22 °C ± 2.5 °C, 60 % ± 8.7 % relative humidity and a 16 h photoperiod with additional light (if solar radiation fell below 25 klx, provided by SONT Philips Master Agro 400W). Plants were watered by hand as required on a daily basis. In addition, plant protection was performed by spraying against thrips and spider mites according to horticultural practice. For RNA isolation plant material was harvested after seven days of cultivation in either γARD or ARD soil. The whole root system was cut from the individual plants, washed with water, put into 2 ml tubes (Sarstedt, Nümbrecht, Germany) and frozen in liquid nitrogen. Until homogenization of plant material, samples were stored at -80 °C. Furthermore, the main shoot length of the remaining 5 plants was measured weekly.

RNA extraction

For each of the two variants (γARD or ARD), roots of 5 individual plantlets each were combined in a liquid nitrogen cooled steel cup to form one pooled biological replicate (Supplementary Fig. S1). As a criterion for the combination of different replicates, equal average shoot length of the plants in the pool was used. In total five pools (= biological replicates) per variant were generated. The pools were homogenized and cells were disrupted using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) by adding steel beads (ø 1.5 cm) cooled in liquid nitrogen to the cups which were then subsequently shaken in the mixer mill for one minute at 30 Hz. RNA extraction of the samples was achieved by using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) with an extraction buffer for phenol containing plants according to the manufacturer's instructions. In total 100 mg of the homogenized material was weighed into 2 ml tubes (Sarstedt, Nümbrecht, Germany) to obtain adequate RNA yield and quality, both measured by a spectrophotometer (Nanodrop 2000c, Peqlab, Erlangen, Germany). Afterwards, genomic DNA was removed via in solution DNA digestion using 1 µg of total RNA and DNase I (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The resulting RNA was checked for integrity on a 1 % agarose gel. Aliquots of 1 µg total RNA were stored at -80 °C until further proceeding.

Massive analysis of cDNA ends (MACE)

Transcriptomic analysis of root samples was accomplished by means of the MACE technology (Kahl et al. 2012). Extracted RNA of two biological replicates per variant (see section: *RNA extraction* and Supplementary Fig. S1) was sent for analysis to GenXPro GmbH (Frankfurt am Main, Germany). After sequencing raw data was processed using CLC Genomics Workbench 8 (Qiagen, Hilden, Germany). Raw sequences obtained in this study have been submitted to the NCBI Sequence Read Archive (SRA) under the accession number SRP077963. Using a poly-A adapter raw sequences were trimmed to remove sequenced bases resulting from cDNA synthesis primers. In addition, the adapters allowed a further quality check. Trimmed sequences of the MACE analysis were mapped to the *Malus x domestica*.v1.0-primary.mRNA database obtained from https://www.rosaceae.org (11.11.2014). Parameters for mapping were as follows: Mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, strand specific = both, maximum number of hits for a read = 10 and expression values = total counts. CLC's implemented Baggerley's test – which accounts for transcript reads (= counts) according to the sample specific total number of sequence reads (= total counts) – was used to identify differentially expressed or unique genes with a false discovery rate (FDR) corrected p value < 0.05 according to the ratio of ARD to γARD of trimmed read numbers (ARD γARD^{-1}). Ratios between 0 and 1 were transformed by multiplying with the negative reciprocal value. Negative resulting values indicated a downregulation of specific transcripts in ARD samples compared to γARD samples, whereas positive values specified an upregulation of transcripts in ARD samples. All genes were processed in MapMan (Thimm et al. 2004) to classify affected metabolic

pathways. Corresponding sequences were blasted using default parameters for BLASTN of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to obtain potential gene functions. From the list of differentially expressed or unique genes with a FDR adjusted p value < 0.05 and at least 2-times regulation, candidate genes with reported function in biotic stress response and plant defense were selected to be further evaluated in subsequent experiments by RT-qPCR (Table 1). In addition, the phytoalexin biosynthesis genes biphenyl synthase 2 and 4 (*BIS2*, *BIS4*) as well as the biphenyl hydroxylase P450 oxidase (*B4Ha*) were included in RT-qPCR experiments as they belong to gene families (*BIS3*, *B4Hb*) identified here for the first time in apple roots.

Primer design

Parameters for primer design included a primer length of 18-24 bp, a resulting fragment length of 100-200 bp, T_M of 57-63 °C, GC content of 40-60 %, no more than triple repeats (e.g. GGG), GC clamp at the 3' end but no more than two times GC in the last five base pairs, no dimers, no self-complementarity and no hairpin structures. Potential primers were checked according to set parameters using Clone Manager 9 Demo (Sci-Ed Software, Denver, CO, USA). To get reverse primers GeneRunner (Gene Runner, http://www.generunner.net/) was used to obtain complementary sequences. If all parameters met the criteria, primers were searched in the *Malus x domestica*.v1.0-primary.mRNA database for specificity. Reference genes were selected according to Perini et al. (2014) and Flachowsky et al. (2010). Primer sequences of genes which showed sufficient amplification efficiency (see section below: *RT-qPCR validation*) are listed in Table 1 including their respective MDP ID (Apple gene identification number).

First strand cDNA synthesis

RNA samples were used for first strand cDNA synthesis by employing the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions with 1 µg RNA. Random hexamer primers were included in the reaction mix to reversely transcribe the total RNA. After cDNA synthesis samples were aliquoted for subsequent RT-qPCR experiments and stored at -80 °C.

RT-qPCR validation

Primer pairs of reference and candidate genes were tested in RT-qPCR efficiency tests to check for sufficient amplification efficiency and specificity using a real-time PCR cycler (CFX Connect™, Bio-Rad, Hercules, CA, USA). The efficiency tests were run by combining equal amounts of γARD and ARD cDNA. Mixed cDNA samples were analyzed in a dilution series of 1:10, 1:50, 1:100, 1:500 and 1:1000. For each primer combination (200 nM for each forward/reverse primer) the dilution samples were tested with two technical replicates using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). All primer pairs were tested at 60 °C annealing temperature. The protocol for the

efficiency tests and subsequent RT-qPCRs was as follows: Three minutes at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The program was ended after a melt curve analysis from 65 °C to 95 °C with an increment of 0.5 °C for 5 s at each step. Data was recorded with the Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA) and amplification efficiencies were calculated. Primer pairs with amplification efficiencies of 90-110 % showing specific amplification according to the melt curve analysis were used for ensuing experiments. The 1:10 cDNA dilution was used and data was again recorded with the Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA) for individual samples using five biological replicates (as defined in section: *RNA extraction*) and two technical replicates per variant. Relative normalized expression was calculated using γARD as the denominator according to the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The potential reference genes were tested according to their stability, based on the calculation of Vandesompele et al. (2002). The following reference genes were selected for normalization: actin-7 (*ACT7)*, tubulin beta chain (*TUBB)*, ubiquitin-conjugating enzyme E2 10-like (*UBE210)*, elongation factor 1-alpha (*EF1a)* and elongation factor 1-beta 2-like (*EF1b)*.

Statistical analysis

Statistical analysis was performed using the statistics program R 3.3.2 (R Development Core Team 2008). Means of parameters measured or calculated for γARD were compared to the means of ARD using the Welch Two Sample t-Test (Welch 1947) at a p value level of 0.05. For internal verification, MACE and RT-qPCR datasets were compared by calculating the Pearson product-moment correlation coefficient (Pearson 1895) in Microsoft Excel 2010 to test for reliability of both methods.

Results

ARD soil affected shoot and root growth negatively

Cultivating apple rootstock 'M26' in either γARD soil or ARD soil led to significant shoot length differences already after two weeks (Fig. 1a). Plants grown on ARD soil were 2.9 ± 0.4 cm in size at this time point, whereas the shoot length of γARD plants measured 3.8 ± 0.5 cm. The differences between the two variants increased over time so that γARD plants showed almost double the size of ARD plants at the end of the experiment (Fig. 1a, c) with 28.3 ± 2.3 cm compared to 15.8 ± 2.3 cm. At the final evaluation, also the root system of ARD plants was clearly affected not only in size but displaying darker coloration, too (Fig. 1d). In contrast, plants harvested after one week of cultivation for transcriptomic analysis of early molecular responses to ARD did not show any visual growth differences (Fig. 1b).

Sequencing and mapping

MACE was employed to obtain a detailed view on molecular responses of apple roots in ARD soil. For this, RNA was extracted from roots of plants cultivated in either disinfected replant soil (γARD)

or untreated replant soil (ARD). Samples of cDNA were analyzed and resulted in 12 to 22 million raw reads with an average length of 92.0 to 92.4 bp per sample (Table 2). After trimming of adapter sequences the average length of reads ranged from 86.2 to 87.9 bp. Mapping to the *Malus domestica* sequence database resulted in roughly 32 % specifically mapped reads and about 2 % of reads matching to more than one sequence per sample (Table 2). Reads mapped to more than one sequence (non-specifically mapped reads) were not included in further analyses.

Transcriptomic response of apple 'M26' roots to ARD

In total 1874 differentially expressed or unique genes with a FDR corrected p value < 0.05 could be mapped to the *Malus domestica* database but assigning gene functions was only successful for 1542 genes which are listed in Supplementary Table S1. For an overview of the affected metabolic pathways, expressed transcripts were further analyzed using the MapMan software (Thimm et al. 2004) and the genes were ordered according to their function in the different metabolic pathways (Fig. 2). Regarding all differentially expressed genes, transcripts that could be annotated to the RNA and protein functional categories were most represented but also signaling, transport and stress associated genes were higher represented in relation to the other functional categories (Fig. 2). More transcripts were assigned to these MapMan functional categories in γARD samples in comparison to ARD samples but ARD samples revealed a higher number of genes assigned to secondary metabolism – especially flavonoid, phenylpropanoid and phenolic metabolism – redox reactions and development (Fig. 2). In contrast, genes involved in the primary metabolism were observed to be of lower abundance in ARD samples including genes attributed to cell, cell wall, minor CHO metabolism and photosynthesis. In more detail, Table 3 lists genes with at least 3-times regulation (p_{FDR} <0.05) or which were found only in either γARD or ARD samples. Especially, genes which show the highest upregulation under ARD conditions are interesting candidates to further characterize the reaction of apple roots in ARD and a subset of those has been selected in this study for the RT-qPCR analyses.

Validation of MACE data via RT-qPCR

MACE was performed to generate an overview of ARD affected molecular responses that resulted in the observed severe growth depression. Highly regulated genes might play a prominent role in ARD response and the observed growth depressions in untreated ARD soil point to biotic stress reaction. Hence, the selection of candidate genes to validate the MACE data via RT-qPCR and to analyze their expression in further plant pools was mainly focused on metabolic pathways of stress responses as well as the observed strong changes in secondary plant metabolites together with higher expression in ARD samples.

Calculating the Pearson product-moment correlation coefficient was performed for internal verification of MACE data and revealed a strong correlation between the two datasets with a coefficient of correlation R value of 0.88 ($p < 0.001$). For RT-qPCR experiments actin-7 (*ACT7)*, tubulin beta chain (*TUBB)*, ubiquitin-conjugating enzyme E2 10-like (*UBE210)*, elongation factor 1-

alpha (*EF1a)* and elongation factor 1-beta 2-like (*EF1b)* were used as reference genes according to their expression stability. The investigated genes were ordered according to the following classes: Phytoalexin biosynthesis, plant defense, regulatory function and signaling (Table 4). After testing of all genes in RT-qPCR experiments, two of the genes did not show a significant difference and were expressed equally in both variants. These genes included the caffeic acid 3-*O*-methyltransferase-like (*OMT1a)* gene involved in phytoalexin biosynthesis and the regulatory gene senescence-associated carboxylesterase 101-like (*SAG101)*.

Most interestingly, genes responsible for phytoalexin biosynthesis were 2.1 to 4.1 times upregulated in ARD samples. Here, the biphenyl synthases 2 to 4 (*BIS2* to *BIS4)* showed the highest regulation ranging from 3.7 to 4.1, whereas the caffeic acid 3-*O*-methyltransferase-like *(OMT1b)* gene was 2.1 times upregulated in ARD samples (Table 4). The biphenyl hydroxylase P450 oxidases (*B4Ha* and *B4Hb*) were upregulated 2.6 to 2.8 times. The expression levels were highest for *BIS3* and *OMT1b*, whereas *BIS2* and *BIS4* showed the lowest expression levels among the phytoalexin biosynthesis genes. Also two other genes functioning in overall plant defense, the acidic endochitinase-like (*CHIA)* and the thaumatin-like protein 1a (*TL1)* were upregulated 2.8 and 4.3 fold, respectively, but *CHIA* was higher expressed (Table 4). Similarly, another endochitinase was upregulated 3.3 fold in ARD samples (Table 3).

Genes with regulatory functions showed diverse responses in transcription. The heat shock protein genes 18.5 kDa class I heat shock protein-like as well as 17.1 kDa and 17.3 kDa class II heat shock protein-like (*HSP18.5, HSP17.1* and *HSP17.3)* were 3.4 to 3.9 times downregulated in ARD samples with *HSP17.3* showing the highest expression level, whereas the F-box/kelch-repeat protein *(KFB)* gene was upregulated 6.3 fold in ARD samples but it displayed very low expression. Also many other HSP genes were downregulated 3.1 to 5 fold in ARD samples (Table 3).

Differing regulation was also observed for the selected genes involved in signaling. The genes for ethylene-responsive transcription factor RAP2-11-like (*ERF RAP2.11*: 12.9 times), the gibberellin-regulated protein 1-like (*GASA1*: 2 times) and the LRR receptor-like serine/threonine-protein kinase MRH1 (*MRH1*: 1.4 times) were all downregulated in ARD samples (Table 4). Likewise, several other kinases were downregulated 3.1 to 5.8 fold as well, in addition to another *ERF RAP2.11* gene which was downregulated 3.6 fold (Table 3). In contrast, the genes for dof zinc finger protein DOF3.5-like (*DOF3.*5: 1.4 times), the NAC transcription factor 25-like (*NAC25*: 2 times), the ethylene-responsive transcription factor 1B-like (*ERF1B*: 3.3 times), the zinc finger protein ZAT12 (*ZAT12*: 4.1 times) and the GATA zinc finger domain-containing protein 10-like (*GATAD10*: 15 times) were all upregulated in ARD samples. Regardless of their high regulation, genes *ERF RAP2.11* and *GATAD10* showed very low expression levels, and likewise, overall all upregulated genes in the category signaling displayed minimal expression levels.

Discussion

This study aimed to uncover and investigate the molecular responses of apple roots when faced with ARD soil. For this, the rootstock 'M26' was used as it was already established as a reliable indicator test plant to detect ARD in soils due to its high susceptibility (Yim et al. 2013). The ARD sensitive genotype 'M26' was employed rather than an ARD tolerant genotype such as 'G41' (Fazio et al. 2005). The reason behind this was to uncover ARD affected molecular reactions in roots of a challenged genotype. The identified candidate genes should be analyzed in the next steps in a comparison of sensitive and more tolerant genotypes. Thereby, in later stages mechanisms of tolerance can be identified and molecular markers can be developed enabling breeding for ARD tolerance.

Although no differences in shoot length or plant biomass could be visualized after one week of cultivation when roots were harvested for RNA isolation, plants showed significant growth depressions already after two weeks of cultivation in ARD soil compared to the γ-irradiated ARD soil (Fig. 1). Mazzola and Manici (2012) reported that replant disease symptoms can be observed on a consistent basis one to three months after planting in the field. Under controlled, growth promotive greenhouse conditions in smaller soil volumes symptoms can already be visualized within two to five weeks (Yim et al. 2013, 2015). As we wanted on the one hand to identify early affected molecular reactions before significant growth differences were observable and on the other hand the stress reactions due to the repotting of the plants should not interfere with the reactions to the soil treatments, the harvest time point was set to seven days after planting. If potential candidate genes can be used as markers for replant disease the earlier time point can decrease the cost for breeders and tree nurseries in either waiting to identify more tolerant genotypes or testing of soil for incidence of ARD, respectively. In addition to stunted growth, a smaller sized root system and rotten roots were monitored at the end of the experiment in ARD variants (Fig. 1d). In previous studies the effect of ARD on root morphology was covered and it was observed that epidermal cells and cortical tissues are destroyed (Yim et al. 2013), root tip necrosis occur (Mazzola and Manici 2012), lateral root development is reduced (Savory 1966; Hoestra 1968) and functional root hairs are almost completely missing (Caruso et al. 1989). As a consequence, the overall root biomass is reduced (Mazzola and Manici 2012) indications for which were also observed in the present study (Fig. 1d). As shown by several previous studies (Hoestra 1968; Jaffee et al. 1982a; Mai and Abawi 1981; Slykhuis and Li 1985), disinfection of ARD soil improved plant growth stressing the association of ARD with the soil biome resulting in biotic stress. Mainly different soil microbial communities as well as higher amounts of detrimental soil organisms in ARD soil compared to γARD soil must have led to the observed reduced plant growth (Franke-Whittle et al. 2015; Mazzola et al. 2015; Yim et al. 2015).

Yim et al. (2015) assumed that damaged ARD roots invested more energy in defense reactions because in a previous study (Yim et al. 2013) ARD roots showed stronger lignification which may be due to oxidation of phenolic compounds, also an important part of defense responses in plants (Vermerris and Nicholson 2008). The darker coloration of ARD roots (Fig. 1d) leads to the

assumption of higher phenolic concentrations in the root system. Likewise, Henfrey et al. (2015) showed that phenolic compounds accumulated in plants exposed to ARD suggesting a function as antioxidant substances. In addition, the flavonoid phloridzin was found to a higher extent in root exudates of ARD plants (Hofmann et al. 2009). These findings correlate with the observed changes in molecular responses of ARD roots (Fig. 2). Here, genes involved in secondary metabolism, especially flavonoid, phenylpropanoid and phenolic metabolism were upregulated in ARD samples.

Overall, the MACE technology was successfully employed for the first time working with apple root material. Parameters shown in Table 2 were within ranges already reported for other studies using MACE: The number of reads with 12 to 22 millions was higher compared to data obtained for tomato pollen with 3 to 6 million reads (Bokszczanin et al. 2015) and similar to tomato leaves with 12 to 15 million reads (Fragkostefanakis et al. 2015). In the aforementioned studies the percentage of mapped reads ranged between 30 to 45 % for tomato pollen which is comparable to our study whereas in tomato leaves only 10 to 11 % of all reads could be mapped. The average length of reads was in the previously reported range of 50 to 100 bp (Kahl et al. 2012; Müller et al. 2014; Zajac et al. 2015). Furthermore, the validation of MACE data via RT-qPCR revealed that all tested candidate genes at least showed the same tendency of regulation in both methods and a good correlation ($R = 0.88$). A first set of regulated genes identified by MACE which were selected for RT-qPCR experiments comprised different classes. In particular, phytoalexin biosynthesis, plant defense, regulatory function and signaling classes included genes with high differential regulations and were therefore investigated in detail (Table 4). Furthermore, *BIS2*, *BIS4* and *B4Ha* were included in RT-qPCR experiments as they belong to gene families (*BIS3*, *B4Hb*) identified here for the first time in apple roots. All classes had previously been reported in literature to play a role in biotic stress response (see below). Thus, this study contributes to the knowledge of reactions of roots of woody plant species to biotic stressors which is scarce compared to the well-studied responses of herbaceous species in aboveground tissues.

Interestingly, since not expected, the biphenyl synthases *BIS2* to *BIS4*, the caffeic acid 3-*O*-methyltransferase-like *OMT1b* gene and the biphenyl hydroxylase P450 oxidases *B4Ha* as well as *B4Hb* were significantly upregulated in ARD roots (Table 4). The products of these genes function as important enzymes in phytoalexin biosynthesis (Khalil et al. 2015). In cytological studies on the phytoalexin camalexin and its antimicrobial mechanisms in *Arabidopsis* in response to infection with *Botrytis cinerea* and *Alternaria spp.,* scientists found that phytoalexins cause the disruption of membrane integrity (Rogers et al. 1996), inhibition of conidial germination and germ-tube elongation (Sellam et al. 2007), as well as the apoptotic-like programmed cell death of fungi (Shlezinger et al. 2011). The production of phytoalexins is one possibility for plants to cope with biotic stress (Ahuja et al. 2012; Darvill and Albersheim 1984; Kokubun and Harborne 1995; Kuć 1995). Chizzali et al. (2012) monitored the accumulation of the apple phytoalexins aucuparin and dibenzofuran in the transition zone of *Erwinia amylovora* infected apple shoots. Higher expression of genes responsible for the production of phytoalexins correlated with the overall higher amount of metabolites identified

(Chizzali et al. 2012). Interestingly, in the present study *BIS3* showed the highest expression level amongst the tested phytoalexin biosynthesis genes (Table 4), whereas the other genes involved in phytoalexin biosynthesis showed comparable expression levels except for *BIS4* with lower expression levels. *BIS3* was also the gene with the highest expression in the study of Chizzali et al. (2012), although it must be taken into account that the genes were tested in aboveground tissue. Nevertheless, the expression was observed close to the infection site, just as in this study in which the root system was exposed to the biotic stress of ARD. Regarding *BIS4*, Shin et al. (2016) also found an upregulation of this gene, annotated as chalcone synthase, already 24 hours after infection with *P. ultimum* and with increasing time the gene was expressed even higher in infected roots. Due to the potential relevance of this oomycete in ARD the finding of *BIS* genes seems to be enhanced. For the first time, our study showed induced expression of genes involved in the biosynthesis of phytoalexins in apple roots. Detailed biochemical analyses proving the presence of biphenyls and dibenzofurans in roots are in progress (cooperation with L. Beerhues, University of Braunschweig) and revealed increased levels of four different phytoalexins in ARD roots (data not shown). As phytoalexins act predominantly against fungi the role of fungi in ARD seems to be enhanced as suggested in literature (Franke-Whittle et al. 2015; Manici et al. 2013). Rogers et al. (1996) mentioned a majority of cases showcasing the correlation of host resistance and the level of phytoalexin accumulation in potato, *Vicia fabia* and *Phaseolus vulgaris*. Hence, the upregulation of phytoalexin biosynthesis genes resulting in increased levels of phytoalexins was not expected due to the obvious lack of their effect. Nevertheless, Rogers et al. (1996) also reported on *Brassica* spp. and various cruciferous plants that did not show this typical correlation. In addition, the authors noted that phytoalexins in high concentrations may lead to cytotoxicity. Therefore, another explanation may be that the production of phytoalexins in roots of 'M26' in response to ARD led to a situation in which affected plants were not able to handle the sequestration of potentially toxic molecules any longer, killing themselves in the process of trying to respond with a well-established defense. Eventually, it is important to link found gene expression results to the analysis of microbial communities in ARD soils to better understand the etiology of the disease.

Ethylene can induce synthesis of phytoalexins derived from the phenylpropanoid pathway (Chung et al. 2001; Ishigaki et al. 2004; Kamo et al. 2000). Also more generally, ethylene is often reported to play an important role in defense reactions of plants towards biotic stress (Broekaert et al. 2006; Glazebrook 2005). Infecting plants with one of the causal agents of ARD, *P. ultimum*, led to the upregulation of 1-aminocyclopropane-1-carboxylic acid synthases and ethylene responsive transcription factors involved in ethylene biosynthesis in root tissue (Shin et al. 2014). In ARD roots, two *ERF RAP2.11* genes were downregulated while *ERF1B* showed an upregulation in ARD roots. Other genes involved in ethylene biosynthesis and signaling as well as earlier time points need to be investigated to gather a complete picture of their impact in the ARD response.

With regard to ethylene being an important signaling molecule to activate defense related genes such as the pathogenesis-related chitinase gene (Lorenzo et al. 2003; Pré et al. 2008; Punja and Zhang 1993; Shin et al. 2014; Solano et al. 1998), *CHIA* and another endochitinase were analyzed and found upregulated in ARD roots. The same was true for the pathogenesis related gene thaumatin-like 1 (*TL1)* being related to thaumatin with reported antifungal function (Liu et al. 2010). In rice *TL1* gene expression was enhanced by microbial infection with *Rhizoctonia solani* and plant hormones such as ethylene (Velazhahan et al. 1999). According to our transcriptomic data, the well-studied defense mechanism of ethylene production and downstream signaling (e.g. defense gene activation) upon pathogen attack does not seem to work properly in 'M26' plants subjected to ARD.

DNA-binding genes can play a role in defense mechanisms as regulators of defense gene expression upon salicylic acid and oxidative stress signals (Chen et al. 1996; Kang and Singh 2000; Yanagisawa 2002; Zhang et al. 1995). In our study, *DOF3.5* showed an upregulation in ARD roots (Table 4).

Biotic stress triggers internal defense reactions of affected plants by activating signal transduction pathways (Dodds and Rathjen 2010; Tsuda and Katagiri 2010; Sato et al. 2010) often involving transcription factors (Davletova et al. 2005; Li et al. 2014; Nuruzzaman et al. 2013). In rice, zinc finger protein genes of the *GATA* family were repressed in the interaction with the pathogen *Magnaporthe oryzae*. In contrast, we could show an increase of *GATAD10* in ARD roots. Another zinc finger protein gene, namely *ZAT12,* was upregulated in ARD roots as well. In previous studies it was shown that this gene is, amongst other functions, associated with biotic stress response, especially reactive oxygen signaling (Davletova et al. 2005). This form of signaling was highlighted by Baxter et al. (2013) pointing to the importance of reactive oxygen signaling in diverse biological processes including pathogen attacks. Also *NAC* transcription factors play a vital role in defense mechanisms as they function in transcriptional reprogramming associated with plant immune responses (Nuruzzaman et al. 2013). In their review, the authors also mentioned the upregulation of *NAC* genes in response to attack by viruses, fungal elicitors and bacteria. Likewise, in ARD roots, *NAC25* showed higher expression levels (Table 4). Regardless of their upregulation, the analyzed transcription factors obviously did not lead to a sufficient immune reaction.

The same applied to other signaling genes reported in literature. Zhu et al. (2005) implicated gibberellin to be involved in plant immunity by regulating DELLA proteins and therefore modulating the balance of salicylic and jasmonic acid signaling during plant immunity. The gibberellin-regulated protein gene *GASA1* was downregulated in roots cultivated in ARD soil (Table 4). Interestingly, there are several studies that either observed increased resistance or susceptibility by altering DELLA proteins (De Bruyne et al. 2014). *MRH1* and several other kinases were downregulated in roots cultivated in ARD soil as well (Table 3 and 4). Kinases such as *MRH1* lead to further signal transduction pathways including phytohormone and reactive oxygen signaling conferring disease resistance (Afzal et al. 2008). The downregulation of these signaling genes in ARD roots might be an

indication of an impaired defense reaction in ‘M26’ plants being obviously not sufficient to protect the plant from the biotic stress.

The last class of tested genes included regulatory genes which also play an important role in biotic stress response to maintain a balanced metabolism even when faced with adverse situations. These genes include F-box proteins (Ul Hassan et al. 2015) and heat shock proteins (Park and Seo 2015) that regulate overall protein function also upon biotic stress. Here, *KFB* was upregulated in ARD samples whereas the heat shock protein genes *HSP18.5*, *HSP17.1* and *HSP17.3* as well as several other HSP genes were downregulated (Table 4).

Ul Hassan et al. (2015) highlighted the functions of F-Box proteins and their involvement in phytohormones signaling as well as pathogen defense. But it was also reported that F-Box proteins interact with enzymes in the secondary metabolism, especially in the phenylpropanoid metabolism. However, the authors also stated that *KFB* genes could actually promote successful infection of pathogens in the plant. The upregulation of *KFB* in ARD roots therefore suggests either that it could have had an influence on the higher expression of secondary metabolism genes (Fig. 2) and/or that potential harmful microorganism established themselves in the root system more easily.

The investigated heat shock protein genes were downregulated in ARD samples. Due to their function in protein stabilization and refolding of proteins under stressful conditions (Hüttner and Strasser 2012; Sitia and Braakman 2003; Whitley et al. 1999) but also their involvement in innate immunity (Li et al. 2009; Liu and Howell 2010; Nekrasov et al. 2009), their observed downregulation again points to impaired biotic stress response under ARD conditions and highlights the importance of these genes in maintaining metabolism and growth.

This lays a foundation on which future projects should gain valuable knowledge regarding molecular effects in roots of ARD affected plants. It is the first time a study deals with the reaction to the whole microorganism complex leading to ARD on the molecular level in planta. A different approach was used in the most recent study by Shin et al. (2016) which investigated the interaction of one potential causal agent of ARD, *P. ultimum*, with the plant root. Their approach showed the specific molecular response of roots to this pathogen and identified genes involved in the early pathogen detection as well as the infection-induced production of pathogenesis-related proteins and several antimicrobial secondary metabolites. Moreover, it became obvious that ethylene, jasmonate and cytokinin signaling seem to play a role in the defense response (Shin et al. 2016). Genes encoding for enzymes involved in cell wall modification and antioxidant production were found upregulated in apple roots inoculated with *P. ultimum* (Shin et al. 2016). Thus, several similar observations as in the present study were reported in that one of Shin et al. (2016), but only relatively few differentially expressed genes (e.g. *BIS4*) could be detected by both approaches. This is partly due to the different genotypes and time points analyzed, but also points to the value of both approaches. The complex interactions of the different causal agents and other microorganisms in ARD affected soil cannot be covered by investigations involving single pathogens. From the synopsis of the reactions of roots to

particular pathogens on the one hand and the observed transcriptomic changes in reaction to proven ARD soils (this study) should result in a better understanding and finally a complete picture of the ARD problem.

To complement our experimental approach employing ARD soils detailed analyses of the microbial communities in ARD soil and disinfected ARD soil are required. Since the ARD causes were shown to differ between different sites and soil types future studies need to involve other ARD soils to discriminate between site- or soil-specific and common ARD responses.

In conclusion, this study highlighted the importance of unraveling the molecular responses of 'M26' to ARD. For the first time, gene expression was investigated in roots of ARD affected plants with a detailed investigation of genes involved in phytoalexin biosynthesis as well as plant defense, regulatory functions and signaling. Further in depth analysis of these genes, particularly, those found in the secondary metabolism also in a time course experiment should improve the understanding of ARD and the physiological responses of plants towards ARD. The maximal number of differentially expressed genes was observed 48 h after inoculation with *P. ultimum* (Shin et al. 2016). Therefore, earlier time points should be included in future experiments. The novel finding of upregulated phytoalexin biosynthesis genes raises future research questions addressing their effects on the microorganisms involved in ARD. It will also be interesting to clarify if they are exuded by the roots and thereby affecting the rhizosphere microbial community, since a recent study identified bacterial degraders of phenolic compounds to be less abundant in ARD soils (Yim et al. 2015). Taking all the findings into consideration, it seems that typical defense reactions towards biotic stress found in literature take place in ARD affected plants as well, but they did not allow responding to the biotic stressors attack adequately leading to the observed growth depressions in ARD variants. Furthermore, other plant tissues in order to differentiate between local and systemic reactions as well as other ARD soils and apple genotypes should be analyzed in order to better understand ARD on a molecular level and assist in breeding.

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2.2. Figures and tables

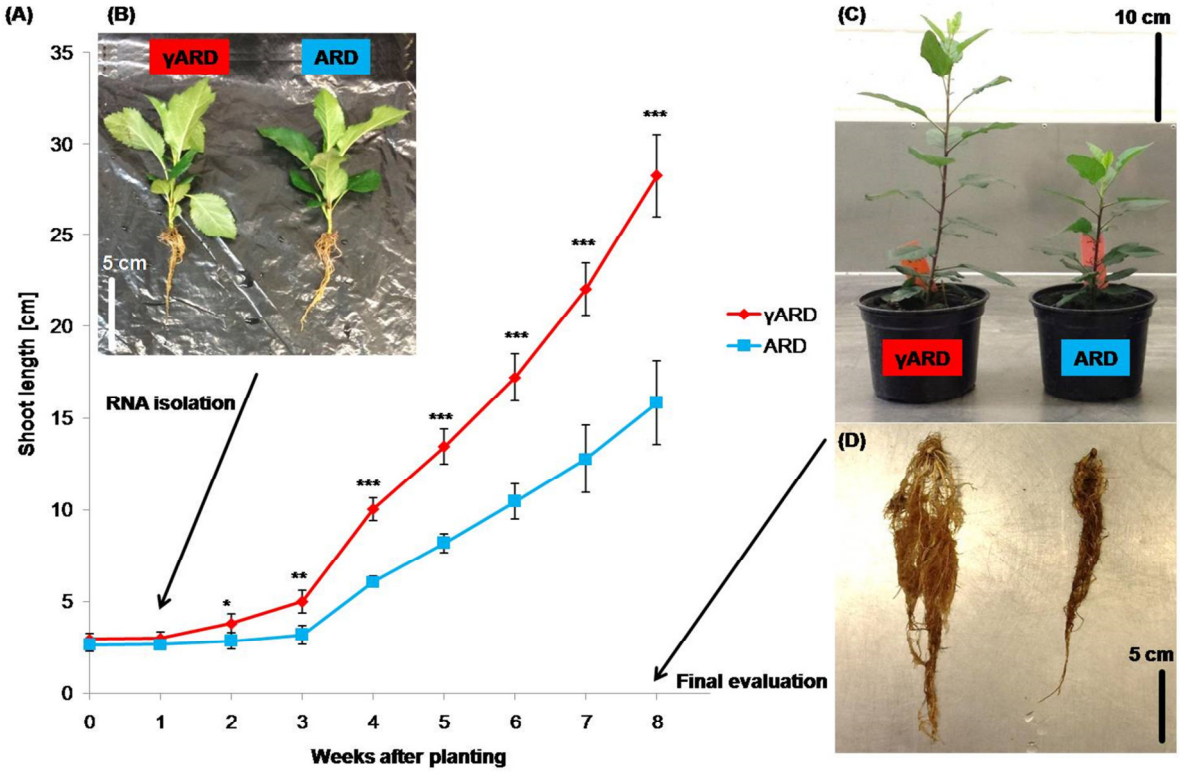


Fig. 1 Response of 'M26' plants to γARD and ARD soil. **a** Shoot length development over time. Differences between plants were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). **b** Overall plant habitus after one week at the time of sampling for RNA isolation and (**c**) eight weeks of cultivation. **d** Root system after eight weeks of cultivation.

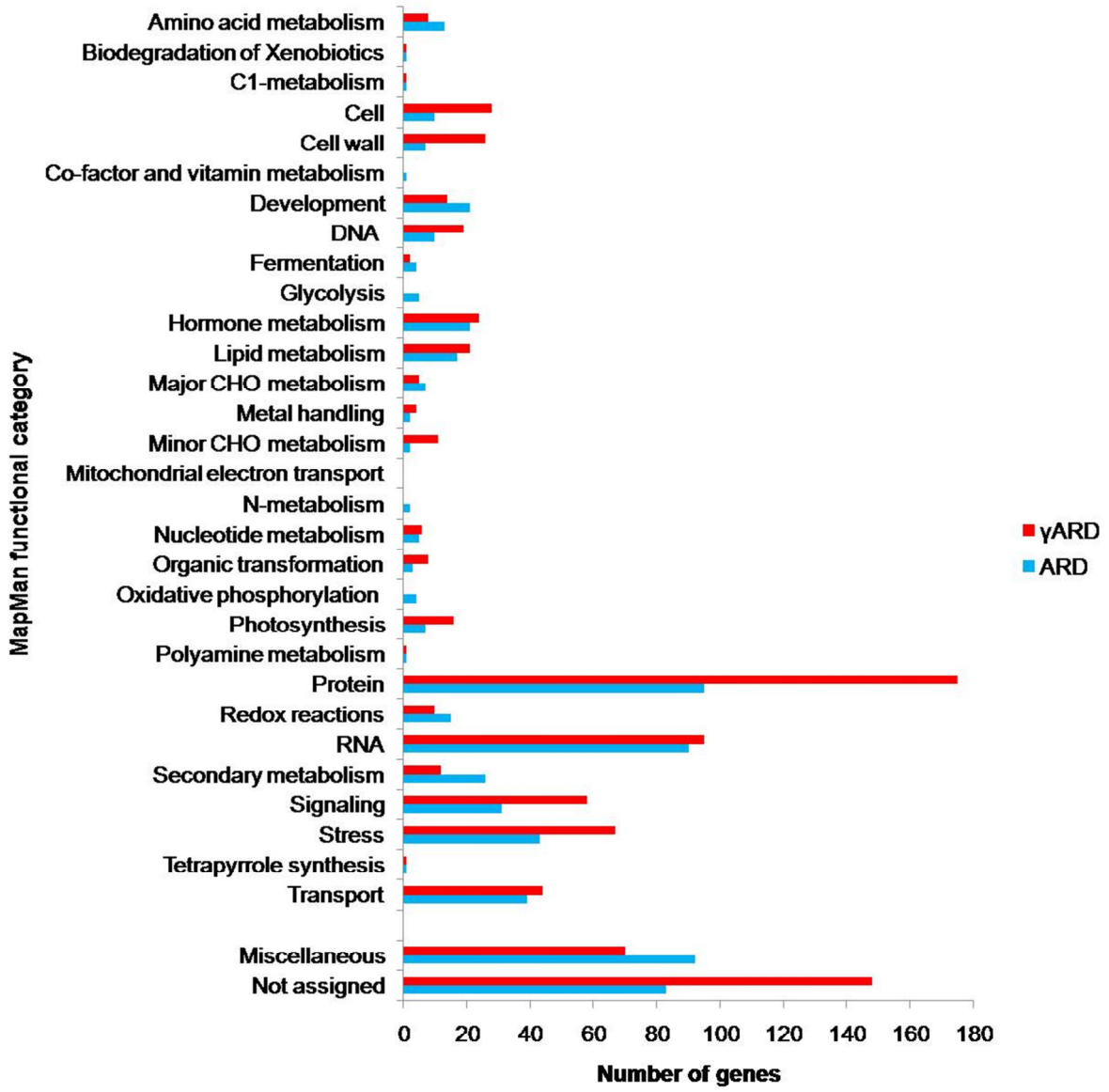


Fig. 2 Distribution of differentially expressed and unique genes with a FDR corrected $p < 0.05$ in γARD (red color) and ARD (blue color) samples according to MapMan functional categories.

Table 1 Primer sequences for genes analyzed by RT-qPCR.*

Gene name (MDP ID)	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
actin (MDP0000921834)	*ACT*	f: TACCATGTTCCCTGGCATTG r: ACCTGCTGGAAAGTGCTAAG	156	92.7	0.995
actin-7 (MDP0000774288)	*ACT7*	f: TGAGTCACACTGTGCCAATC r: TTTCCCGTTCAGCAGTAGTG	147	93.3	0.999
elongation factor 1-alpha (MDP0000304140)	*EF1a*	f: GAACGGAGATGCTGGTATGG r: CCAGTTGGCTCCTTCTTCTC	159	103.0	0.998
elongation factor 1-beta 2-like (MDP0000903484)	*EF1b*	f: GAGAGTGGGAAATCCTCTG r: ACCAACAGCAACCAATTTC	138	100.6	0.998
glyceraldehyde-3-phosphate dehydrogenase (MDP0000645828)	*GAPDH*	f: TGAGAAGGCTGCAACCTATG r: GACTCCTCCCTGTTCATGAC	176	90.3	0.998
ribulose bisphosphate carboxylase/oxygenase activase (MDP0000321244)	*RCA*	f: GTGACGGTCGTATGGAGAAG r: CCTGGGAAGGTGTCAACAAG	130	99.0	0.997
tubulin alpha-2 chain (MDP0000832105)	*TUBA*	f: AGGAAGACGCCGCTAATAAC r: CAAACGGGAATCAACGGAAAG	121	105.1	0.997
tubulin beta chain (MDP0000951799)	*TUBB*	f: TTCTCTGGGAGGAGGTACTG r: GTCGCATTGTAAGGCTCAAC	147	96.9	0.997
ubiquitin-conjugating enzyme E2 10-like (MDP0000140755)	*UBE210*	f: TGCCCTCGTGTACTGATTTG r: GCAGATGATGACGGTGTTTG	105	94.0	0.998
17.1 kDa class II heat shock protein-like (MDP0000700383)	*HSP17.1*	f: CGCCGAGAAGTCATTCAAC r: CACGAACACGTAGGAGTTAG	109	92.8	0.999
17.3 kDa class II heat shock protein-like (MDP0000548065)	*HSP17.3*	f: GACATGCCGGGACTGAAG r: CATTCTCCGGCAACACAAAC	169	94.7	0.998
18.5 kDa class I heat shock protein-like (MDP0000759666)	*HSP18.5*	f: GCTTAAGATCAGCGGAGAG r: CTTCGGAACAGTGACACTTAG	175	98.2	0.997
acidic endochitinase-like (MDP0000218691)	*CHIA*	f: CTGAGTGCTGACATCAGAAC r: GACTGACCACCTAAGAAGTTG	149	99.5	0.999
biphenyl synthase 2 (MDP0000716308)	*BIS2*	f: CTTGTGGTTTGCGCAGAG r: GTCTGCATGCCACGATTTC	172	96.8	0.997
biphenyl synthase 3 (MDP0000287919)	*BIS3*	f: GGCAAGAAGCAGCATTGAAAG r: CACAACCTGGCATGTCAAC	105	95.3	0.998
biphenyl synthase 4 (MDP0000302905)	*BIS4*	f: CAACAAGCAGCACTGAAAGC r: GATGGGTTAAGGCCAAGGAG	140	102.0	1.000
caffeic acid 3-O-methyltransferase-like (MDP0000456277)	*OMT1a*	f: CAGGCGTCAGGTGAAAGATG r: GGGTGCTATGGTTGAACATTG	140	95.9	0.999
caffeic acid 3-O-methyltransferase-like (MDP0000745475)	*OMT1b*	f: CATCTGAGATCGCAGCTCAC r: GTAGAGCCTCCGGAAACAC	146	99.7	1.000
cytochrome P450 CYP736A12-like (MDP0000205306)	*B4Ha*	f: GATGTGCACGCTCGAATTAC r: ATTGGCGTCCTCTTGAACAG	106	99.1	0.999

Gene name (MDP ID)	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
cytochrome P450 CYP736A12-like (MDP0000152900)	*B4Hb*	f: GCTGAGTATGGCCCGTATTG r: AGGAACCCGTCGATTATTGG	156	100.0	0.999
dof zinc finger protein DOF3.5-like (MDP0000308863)	*DOF3.5*	f: CTTTCAGCATCACAACTCATC r: GCAAACACAACAGCCAAATC	105	98.7	1.000
ethylene-responsive transcription factor 1B-like (MDP0000127134)	*ERF1B*	f: GTCACCTGAATCTTCGTTTG r: GGAAATCAGACCGTAGAGAAG	121	99.7	0.997
ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)	*ERF RAP2.11*	f: TTCCAACAGCCGAAGCAAG r: CTTTGATCTCAGCAACCCATCTC	169	96.4	0.997
F-box/kelch-repeat protein (MDP0000708634)	*KFB*	f: GACCTTGCCCACAATCCTC r: GGTGGGTGTAAAGGCTGAAG	145	96.5	1.000
GATA zinc finger domain-containing protein 10-like (MDP0000922823)	*GATAD10*	f: GCTCGTTTCTGGAGGAGTC r: GATTCCCGCTGTCGTAGAATC	153	99.6	0.998
gibberellin-regulated protein 1-like (MDP0000140078)	*GASA1*	f: CGTTGCAGCTGTGTTCCTC r: CATCTGCATGCCCGAATATGAG	156	101.9	0.997
LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	*MRH1*	f: CGAGGTTTCATGGTTGTTG r: CGCAGAATGACAACAGAATC	163	100.1	0.998
NAC transcription factor 25-like (MDP0000842702)	*NAC25*	f: CGTTGCCACAACCACTAC r: CTCCCATGTCATCTGATATTC	199	100.8	0.995
senescence-associated carboxylesterase 101-like (MDP0000837935)	*SAG101*	f: CGTAAACTAGCGATGCAGAAG r: GGACTCCTCCTTGTACCATTC	102	102.1	0.999
thaumatin-like protein 1a (MDP0000552328)	*TL1*	f: ACGGCTTCAACTTGCCTATG r: GCCGCTTTCACTTGAAGTTG	118	92.5	0.992
zinc finger protein ZAT12 (MDP0000595671)	*ZAT12*	f: CAAGAAGCCGAAGCTAATGC r: GTCCGACCGTGAACTCAAG	125	99.2	0.997

*All primers (f: forward, r: reverse) were evaluated in an amplification efficiency test at an annealing temperature of 60 °C. Grey-shaded genes were examined as potential reference genes.

Table 2 Sequencing results of the MACE analysis for both pools of γARD and ARD samples.*

Sample	Number of raw reads	Average length before trimming [bp]	Reads after trimming	Average length after trimming [bp]	Non-specifically mapped reads [number | % trimmed reads]		Unique mapped reads [number | % trimmed reads]	
γARD 1	12,343,547	92.2	12,338,086	87.6	266,108	[2.16]	3,919,214	[31.77]
γARD 2	17,045,051	92.0	17,034,304	86.2	428,165	[2.51]	5,214,009	[30.61]
ARD 1	21,676,247	92.4	21,668,842	87.4	439,672	[2.03]	6,867,552	[31.69]
ARD 2	16,847,113	92.4	16,840,977	87.9	338,234	[2.01]	5,329,115	[31.64]

*Sequences were trimmed employing poly-A adapter sequences using CLC Genomics Workbench 8. Trimmed sequences were mapped to the predicted transcriptome of *Malus domestica*. Reads mapped to more than one sequence (non-specifically mapped reads) were not included in further analyses.

Table 3 List of genes with a FDR corrected p < 0.05 and at least 3-fold regulation or which were found only in either γARD or ARD samples.*

MapMan functional category	MDP ID	Name	Normalized transcript abundance γARD ± SD	Normalized transcript abundance ARD ± SD	Ratio (ARD/γARD)
Biodegradation of Xenobiotics	MDP0000267976	glyoxal oxidase-related	21.65 ± 5.41	6.97 ± 1.89	-3.11
Cell	MDP0000158089	protein binding / structural molecule	0.22 ± 0.15	0.83 ± 0.33	3.78
Cell wall	MDP0000220329	ADPG1; polygalacturonase	0.22 ± 0.19	0.05 ± 0.07	-4.69
Cell wall	MDP0000189708	phytochelatin synthetase-like	0.67 ± 0.14	0.17 ± 0.08	-3.81
Development	MDP0000222217	transducin family protein	0.13 ± 0.07	0.02 ± 0.03	-5.57
Development	**MDP0000837935**	**senescence-associated carboxylesterase 101-like**	**0.1 ± 0.03**	**0.36 ± 0.01**	**3.66**
Development	**MDP0000842702**	**NAC transcription factor 25-like**	**0.16 ± 0.11**	**0.88 ± 0.01**	**5.60**
Development	MDP0000258530	ATAF1; transcription activator/ transcription factor	0.03 ± 0.04	0.21 ± 0.04	7.19
Development	MDP0000209665	transducin family protein	0.03 ± 0.04	0.25 ± 0.1	8.54
DNA	MDP0000315404	histone H3.2	0.26 ± 0.2	0.83 ± 0.26	3.17
Fermentation	MDP0000374885	ALDH2B7; 3-chloroallyl aldehyde dehydrogenase	1.23 ± 0.33	0.38 ± 0.21	-3.21
Hormone metabolism	MDP0000229843	leucoanthocyanidin dioxygenase-like	2.38 ± 1.21	7.54 ± 1.09	3.16
Hormone metabolism	**MDP0000127134**	**ethylene-responsive transcription factor 1B-like**	**0.2 ± 0.05**	**1.12 ± 0.01**	**5.63**
Lipid metabolism	MDP0000827665	glycerophosphodiester phosphodiesterase GDE1-like	0.28 ± 0.06	0 ± 0	only γARD
Miscellaneous	MDP0000616265	TT7 (TRANSPARENT TESTA 7)	0.13 ± 0.07	0 ± 0	only γARD
Miscellaneous	MDP0000119148	CYP86B1; electron carrier	0.58 ± 0.01	0.13 ± 0.01	-4.49
Miscellaneous	MDP0000214250	secoisolariciresinol dehydrogenase-like	0.57 ± 0.11	0.13 ± 0.01	-4.40
Miscellaneous	MDP0000265942	14 kDa proline-rich protein DC2.15-like	1.14 ± 0.11	0.29 ± 0.09	-3.87
Miscellaneous	MDP0000179808	GDSL-motif lipase/hydrolase family protein	5.99 ± 0.25	1.63 ± 0.7	-3.68
Miscellaneous	MDP0000624444	dihydroflavonol 4-reductase family	1.26 ± 0.29	0.38 ± 0.14	-3.35
Miscellaneous	MDP0000163387	GDSL-motif lipase/hydrolase family protein	0.92 ± 0.3	0.3 ± 0.16	-3.06
Miscellaneous	MDP0000224878	caffeic acid 3-O-methyltransferase 1-like	0.81 ± 0.11	2.65 ± 0.87	3.29
Miscellaneous	MDP0000514027	peroxidase 5-like	0.71 ± 0.08	2.9 ± 0.51	4.11
Miscellaneous	MDP0000926793	2-methylene-furan-3-one reductase-like	0.7 ± 0.16	3 ± 0.46	4.29
Miscellaneous	**MDP0000152900**	**cytochrome P450 CYP736A12-like**	**0.59 ± 0.42**	**3.23 ± 0.65**	**5.44**
Miscellaneous	MDP0000127122	blue copper protein precursor	0.06 ± 0.08	0.51 ± 0.2	8.71
Not assigned	MDP0000363176	actin-depolymerizing factor 1-like	0.07 ± 0.02	0 ± 0	only γARD
Not assigned	MDP0000325781	chloroplast envelope membrane 70 kDa heat shock-related protein-like	0.31 ± 0.02	0 ± 0	only γARD
Not assigned	MDP0000729669	magnesium transporter NIPA2-like	1.96 ± 0.13	0.25 ± 0.1	-7.82
Not assigned	MDP0000235335	serine/arginine-rich splicing factor RSZ21-like	0.47 ± 0.25	0.08 ± 0.02	-6.21
Not assigned	MDP0000366399	RNA-binding protein 24-A-like	1.68 ± 0.54	0.32 ± 0.13	-5.18
Not assigned	MDP0000951190	protein DUF607	1.19 ± 0.27	0.28 ± 0.06	-4.24
Not assigned	MDP0000234579	QWRF motif-containing protein 7-like	1.26 ± 0.05	0.32 ± 0.06	-4.00
Not assigned	MDP0000135953	1-aminocyclopropane-1-carboxylate synthase (ACS1)	0.68 ± 0.3	0.2 ± 0.11	-3.44
Not assigned	MDP0000147358	abscisic acid receptor PYL2	1.35 ± 0.5	0.39 ± 0.03	-3.44
Not assigned	MDP0000569755	alcohol acyl transferase (AAT)	12.74 ± 3.07	3.71 ± 0.04	-3.43
Not assigned	**MDP0000140078**	**gibberellin-regulated protein 1-like**	**36.85 ± 7.75**	**10.88 ± 0.94**	**-3.39**
Not assigned	MDP0000239264	ubiquitin thiolesterase	1.85 ± 1.05	0.6 ± 0	-3.11

MapMan functional category	MDP ID	Name	Normalized transcript abundance γARD ± SD	Normalized transcript abundance ARD ± SD	Ratio (ARD/γARD)
Not assigned	MDP0000723286	erine/threonine-protein kinase kinX	2.58 ± 0.25	0.84 ± 0.08	-3.08
Not assigned	MDP0000164683	transcription factor SPATULA-like	0.24 ± 0.01	0.76 ± 0.1	3.19
Not assigned	MDP0000194336	UBP13 (UBIQUITIN-SPECIFIC PROTEASE 13)	1.32 ± 0.2	4.33 ± 0.77	3.29
Not assigned	MDP0000902581	OXS3 (OXIDATIVE STRESS 3)	0.32 ± 0.12	1.4 ± 0.3	4.38
Not assigned	**MDP0000922823**	**GATA zinc finger domain-containing protein 10-like**	**0.23 ± 0.09**	**1.05 ± 0.37**	**4.59**
Not assigned	MDP0000884239	PHD finger-like domain-containing protein 5A	0.19 ± 0.15	1 ± 0.18	5.35
Not assigned	MDP0000295606	Agglutinin	0.11 ± 0.07	0.6 ± 0.07	5.47
Not assigned	MDP0000772451	Agglutinin	0.1 ± 0.03	0.72 ± 0.09	7.24
Photosynthesis	MDP0000764147	PPL1 (PsbP-like protein 1)	0.37 ± 0.06	0.05 ± 0.01	-6.97
Photosynthesis	MDP0000613170	glycine cleavage system H protein	2.06 ± 0.16	0.59 ± 0.25	-3.47
Protein	MDP0000478464	MEE49 (maternal effect embryo arrest 49)	0.18 ± 0.09	0 ± 0	only γARD
Protein	MDP0000785092	ATP-dependent Clp protease proteolytic subunit	17.81 ± 5.42	1.08 ± 0.36	-16.41
Protein	MDP0000912900	serine carboxypeptidase S28 family protein	0.47 ± 0.09	0.03 ± 0.04	-15.72
Protein	**MDP0000302779**	**LRR receptor-like serine/threonine-protein kinase MRH1**	**1.64 ± 0.26**	**0.21 ± 0.22**	**-7.65**
Protein	MDP0000291076	60S ribosomal protein L19 (RPL19B)	0.72 ± 0.02	2.67 ± 0	3.73
Protein	MDP0000264532	metalloendoproteinase 1-like	0.07 ± 0.02	0.45 ± 0.04	6.37
Protein	MDP0000053083	60s acidic ribosomal protein P1	0.07 ± 0.02	0.6 ± 0	8.54
Protein	**MDP0000708634**	**F-box/kelch-repeat protein**	**0.21 ± 0.05**	**1.89 ± 0.07**	**9.01**
Protein	MDP0000207838	MMP (MATRIX METALLOPROTEINASE)	0 ± 0	0.3 ± 0.16	only ARD
Redox reactions	MDP0000579840	glutaredoxin family protein	4.9 ± 0.87	1.58 ± 0.28	-3.09
RNA	**MDP0000177547**	**ethylene-responsive transcription factor RAP2-11-like**	**2.68 ± 0.12**	**0.08 ± 0.02**	**-35.29**
RNA	MDP0000228898	S-acyltransferase 1	0.72 ± 0.02	0.16 ± 0.1	-4.35
RNA	MDP0000525933	ethylene-responsive transcription factor RAP2-11-like	2.3 ± 0.18	0.64 ± 0.14	-3.62
RNA	MDP0000142494	NTT (NO TRANSMITTING TRACT)	1.1 ± 0.18	0.32 ± 0.06	-3.48
RNA	MDP0000216027	protein NUC173	1.57 ± 0.27	0.49 ± 0.23	-3.22
RNA	MDP0000319331	LBD11 (LOB DOMAIN-CONTAINING PROTEIN 11)	1.06 ± 0.34	0.33 ± 0.05	-3.18
RNA	MDP0000188637	EDM2; transcription factor	4.26 ± 0.63	1.38 ± 0.2	-3.07
RNA	MDP0000162592	U-box domain-containing protein 21-like	0.49 ± 0.11	1.47 ± 0.07	3.01
RNA	MDP0000153866	dehydration-responsive element-binding protein 2A	1.04 ± 0.1	3.22 ± 1.16	3.08
RNA	MDP0000198116	bHLH093 (beta HLH protein 93)	0.44 ± 0.05	1.45 ± 0.29	3.32
RNA	**MDP0000595671**	**zinc finger protein ZAT12**	**0.94 ± 0.16**	**3.34 ± 0.74**	**3.56**
RNA	MDP0000649022	AP2 domain-containing transcription factor	0.12 ± 0.17	0.67 ± 0.23	5.70
RNA	MDP0000694363	TGACG-sequence-specific DNA-binding protein TGA-1A	0.03 ± 0.04	0.36 ± 0.01	12.36
Secondary metabolism	MDP0000272976	phytoene synthase (PSY)	0.89 ± 0.35	0.15 ± 0.05	-5.87
Secondary metabolism	**MDP0000287919**	**biphenyl synthase 3**	**14.13 ± 6.37**	**64.39 ± 18.61**	**4.56**
Signaling	MDP0000216054	L-type lectin-domain containing receptor kinase S.4	0.44 ± 0.05	0.08 ± 0.02	-5.77
Signaling	MDP0000169530	LRR receptor-like serine/threonine-protein kinase	0.56 ± 0.13	0.1 ± 0.06	-5.65
Signaling	MDP0000155715	kinase family protein	0.82 ± 0.01	0.22 ± 0.14	-3.69
Signaling	MDP0000279287	LOX2 (LIPOXYGENASE 2)	1.56 ± 0.03	0.43 ± 0.11	-3.62
Signaling	MDP0000293592	FRS5 (FAR1-related sequence 5)	1.45 ± 0.44	0.43 ± 0.11	-3.35

MapMan functional category	MDP ID	Name	Normalized transcript abundance γARD ± SD	Normalized transcript abundance ARD ± SD	Ratio (ARD/γARD)
Signaling	MDP0000272246	kinase family protein	1.7 ± 0.58	0.51 ± 0.13	-3.35
Stress	MDP0000214382	HSP21 (HEAT SHOCK PROTEIN 21)	3.19 ± 0.05	0.64 ± 0.44	-5.02
Stress	**MDP0000700383**	**17.1 kDa class II heat shock protein-like**	**8.28 ± 1.4**	**1.7 ± 0.19**	**-4.86**
Stress	**MDP0000548065**	**17.3 kDa class II heat shock protein-like**	**16.39 ± 1.01**	**3.82 ± 0.9**	**-4.29**
Stress	MDP0000254260	heat shock protein 83-like	44.09 ± 0.92	12.09 ± 0.71	-3.65
Stress	MDP0000172108	18.1 kDa class I heat shock protein-like	12.73 ± 2.18	3.52 ± 0.44	-3.62
Stress	**MDP0000759666**	**18.5 kDa class I heat shock protein-like**	**0.37 ± 0.06**	**0.11 ± 0.02**	**-3.48**
Stress	MDP0000185737	TMV resistance protein N-like	0.47 ± 0.09	0.14 ± 0.06	-3.45
Stress	MDP0000621193	17.3 kDa class II heat shock protein-like	53.57 ± 5.61	16.29 ± 3.07	-3.29
Stress	MDP0000197430	ATERDJ2A; heat shock protein binding	1.01 ± 0.18	0.32 ± 0.13	-3.12
Stress	MDP0000211807	glycosyl hydrolase family 18 protein	1.21 ± 0.13	0.4 ± 0.11	-3.02
Stress	MDP0000503944	DNAJ heat shock protein	5.31 ± 1.21	16.95 ± 0.04	3.19
Stress	MDP0000710349	endochitinase-like	1.47 ± 0.33	4.9 ± 1.05	3.34
Stress	MDP0000567268	DNAJ heat shock protein	2.31 ± 1.21	7.86 ± 0.55	3.40
Stress	**MDP0000552328**	**thaumatin-like protein 1a**	**2.88 ± 1.08**	**12.5 ± 1.64**	**4.34**
Stress	**MDP0000218691**	**acidic endochitinase-like**	**0.07 ± 0.02**	**0.53 ± 0.17**	**7.65**
Transport	MDP0000173207	KEA3; potassium ion transmembrane transporter	2.49 ± 0.95	0.73 ± 0.06	-3.40
Transport	MDP0000173864	TMT2 (TONOPLAST MONOSACCHARIDE TRANSPORTER2)	0.78 ± 0.27	0.25 ± 0.1	-3.11

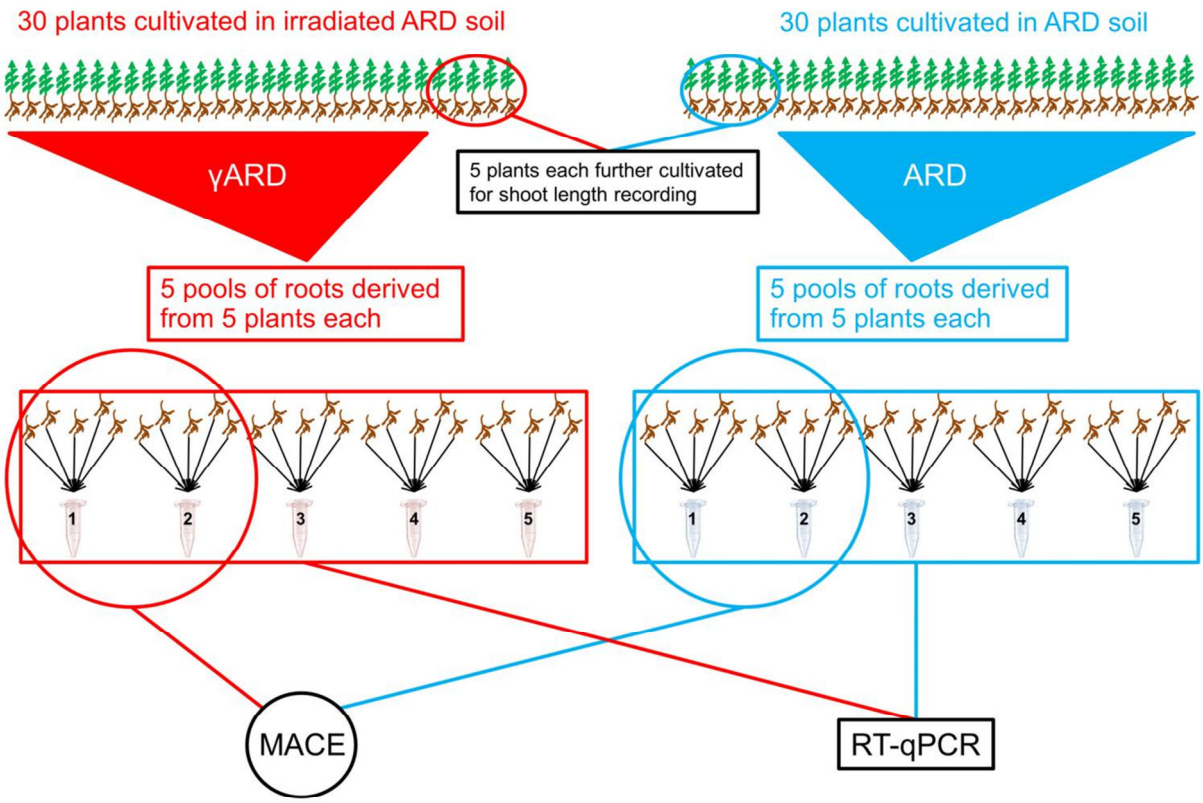
*Ratios are represented by the quotient of ARD/γARD with values between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Bold printing indicates genes that were analyzed in RT-qPCR experiments. Genes are ordered according to their ratios and alphabetically according to their MapMan functional category. Red color indicates downregulation in ARD samples and blue coloring represents upregulation in ARD samples.

Table 4 Expression of candidate genes analyzed by RT-qPCR of γARD and ARD samples.*

Class	Gene	Gene name	Expression γARD ± SD	Expression ARD ± SD	Ratio (ARD/γARD)
Phytoalexin biosynthesis	*OMT1a*	caffeic acid 3-*O*-methyltransferase-like (MDP0000456277)	1.005 ± 0.259 a	1.277 ± 0.171 a	1.271
Phytoalexin biosynthesis	*OMT1b*	caffeic acid 3-*O*-methyltransferase-like (MDP0000745475)	1.892 ± 0.605 a	3.879 ± 0.660 b	2.05
Phytoalexin biosynthesis	*B4Hb*	cytochrome P450 CYP736A12-like (MDP0000152900)	1.394 ± 0.640 a	3.649 ± 0.967 b	2.617
Phytoalexin biosynthesis	*B4Ha*	cytochrome P450 CYP736A12-like (MDP0000205306)	1.072 ± 0.513 a	2.993 ± 0.775 b	2.793
Phytoalexin biosynthesis	*BIS2*	biphenyl synthase 2 (MDP0000716308)	0.742 ± 0.661 a	2.759 ± 0.668 b	3.718
Phytoalexin biosynthesis	*BIS4*	biphenyl synthase 4 (MDP0000302905)	0.114 ± 0.100 a	0.451 ± 0.121 b	3.941
Phytoalexin biosynthesis	*BIS3*	biphenyl synthase 3 (MDP0000287919)	2.081 ± 1.132 a	8.630 ± 1.499 b	4.147
Plant defense	*CHIA*	acidic endochitinase-like (MDP0000218691)	0.349 ± 0.110 a	0.971 ± 0.244 b	2.778
Plant defense	*TL1*	thaumatin-like protein 1a (MDP0000552328)	0.049 ± 0.012 a	0.210 ± 0.101 b	4.288
Regulatory function	*HSP18.5*	18.5 kDa class I heat shock protein-like (MDP0000759666)	0.069 ± 0.028 a	0.018 ± 0.008 b	-3.935
Regulatory function	*HSP17.1*	17.1 kDa class II heat shock protein-like (MDP0000700383)	0.196 ± 0.056 a	0.056 ± 0.014 b	-3.513
Regulatory function	*HSP17.3*	17.3 kDa class II heat shock protein-like (MDP0000548065)	0.721 ± 0.230 a	0.213 ± 0.078 b	-3.384
Regulatory function	*SAG101*	senescence-associated carboxylesterase 101-like (MDP0000837935)	3.508 ± 0.593 a	3.869 ± 0.355 a	1.103
Regulatory function	*KFB*	F-box/kelch-repeat protein (MDP0000708634)	0.004 ± 0.001 a	0.027 ± 0.007 b	6.312
Signaling	*ERF RAP2.11*	ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)	0.040 ± 0.007 a	0.003 ± 0.000 b	-12.933
Signaling	*GASA1*	gibberellin-regulated protein 1-like (MDP0000140078)	0.664 ± 0.110 a	0.338 ± 0.114 b	-1.966
Signaling	*MRH1*	LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	0.053 ± 0.010 a	0.038 ± 0.006 b	-1.381
Signaling	*DOF3.5*	dof zinc finger protein DOF3.5-like (MDP0000308863)	0.017 ± 0.002 a	0.024 ± 0.005 b	1.412
Signaling	*NAC25*	NAC transcription factor 25-like (MDP0000842702)	0.015 ± 0.008 a	0.029 ± 0.005 b	1.937
Signaling	*ERF1B*	ethylene-responsive transcription factor 1B-like (MDP0000127134)	0.009 ± 0.006 a	0.031 ± 0.004 b	3.293
Signaling	*ZAT12*	zinc finger protein ZAT12 (MDP0000595671)	0.017 ± 0.006 a	0.072 ± 0.018 b	4.129
Signaling	*GATAD10*	GATA zinc finger domain-containing protein 10-like (MDP0000922823)	0.002 ± 0.001 a	0.036 ± 0.004 b	15.004

*For normalization *ACT7*, *TUBB*, *UBE210*, *EF1a* and *EF1b* were used as reference genes. Differences in the relative normalized expression of the candidate genes in γARD and ARD samples were tested using a Welch Two Sample t-test (n = 5) with significant differences indicated by different letters ($p < 0.05$) and red (downregulation ARD) or blue (upregulation ARD) coloring. Ratios are represented by the quotient of ARD/γARD with values between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Genes are ordered according to their ratios and alphabetically according to their class.

2.3. Supplementary data



Supplementary Fig. S1 Experimental setup and derived biological replicates. Thirty plants were cultivated in γARD or ARD soil. Five plants per variant were used for shoot length recording and 25 plants per variant were harvested for transcriptomic analyses. In total five pools containing roots derived from five plants each were generated per variant (= biological replicates). Two pools/biological replicates per variant were analyzed with MACE; all replicates were tested in RT-qPCR experiments.

For Supplementary Table S1 see disk in the back of the thesis

Supplementary_Table_S1_Manuscript_I_Chapter_2.pdf

3. Impaired defense reactions in apple replant disease-affected roots of *Malus domestica* 'M26'

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Type of authorship	First author
Type of article	Research article
Contribution to the article	Performed the experiments, data analysis and wrote the manuscript
Co-authors contribution	Benye Liu analyzed the phytoalexin compounds in root material and contributed to writing Dennis Reckwell assisted in analysis of phytoalexin compounds Ludger Beerhues construed phytoalexin data and contributed to writing Traud Winkelmann conceived and coordinated the project, participated in interpretation of data and in writing the manuscript
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3.1. Manuscript

Abstract

A soil- and site-dependent complex of diverse microbial populations causes apple replant disease (ARD), which leads to financial losses for tree nurseries and apple producers due to reduced plant growth and diminished fruit yields. Soil fumigation has been widely used to mitigate ARD, but the use of these chemicals is restricted in the EU. Hence, other counteractions have to be developed. Genomics-based breeding may be used to select ARD tolerant genotypes; however, molecular responses of ARD are not well understood. Recent studies revealed that biotic stress-associated genes involved in typical defense reactions are activated but do not result in an adequate response to ARD. The objective of this study was to analyze selected responsive genes in a time-course experiment to test for expression kinetics and to compare ARD effects on the transcriptomic level between different ARD soils with diverging cropping histories. Cultivating the ARD-susceptible apple rootstock 'M26'on ARD-affected soil resulted in significantly reduced growth already 7 days after planting. The majority of the analyzed genes showed similar transcriptomic responses towards ARD in two different soils. Genes involved in phytoalexin biosynthesis were upregulated in ARD samples already 3 days after planting and reached up to 26-fold changes at day 10, which resulted in high amounts of 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzofuran, 2'-hydroxyaucuparin and noreriobofuran. For the first time, these phytoalexins were detected, identified and quantified in apple roots. The lack of a sufficient defense response may be due to impaired sequestration and/or exudation of the potentially cytotoxic phytoalexins and perturbed formation of ROS, leading to root damage in ARD soils. The findings provide a basis for comparative studies of the defense processes in more ARD-tolerant rootstocks.

Key words: Apple replant disease; biotic stress response; growth depression; *Malus domestica*; plant defense; phytoalexins; quantitative real-time PCR; time-dependent gene expression

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Introduction

Apple replant disease (ARD) describes a severe problem in commercial fruit orchards and tree nurseries with high turnover rates in planting new trees in the same field. Symptoms of the worldwide phenomenon can be detected within one to three months after planting in the field and include diminished growth with restricted and condensed internodes (Mazzola and Manici 2012). In addition to reduced aboveground growth, the biomass of roots is also negatively affected and discolorations as well as root tip necrosis are observed (Mazzola and Manici 2012). Eventually, continuous cropping leads to an adverse influence on fruit yield and quality, demonstrated by undesirable texture, appearance and flavor of apple fruits (Liu et al. 2014).

Many studies established background knowledge concerning potential causal agents in ARD etiology. Although no definitive cause was found, which applies to the majority of cases, parasitic fungi of the genera *Cylindrocarpon* (Tewoldemedhin et al. 2011a; Mazzola and Manici 2012; Manici et al. 2013, 2015; Franke-Whittle et al. 2015), *Phytophthora* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012), *Pythium* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012; Manici et al. 2013) and *Rhizoctonia* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012; Manici et al. 2015) were most often associated with the disease. Abiotic factors may impede apple tree growth but were not found to strongly impact the disease (Spath et al. 2015). Due to the broad spectrum of diverse microorganisms prospectively concomitant to ARD, effective control is nowadays almost not conceivable since soil fumigants, which were successfully used for soil disinfection in former times, are phased out because of environmental concerns (Porter et al. 2010). The EU adopted the directive EC 128/2009 (2009) for the restricted and sustainable use of pesticides. Hence, studies dealing with ARD have focused on finding alternative counteractions for plants to cope with ARD.

Accomplishing better growing conditions for plants faced with ARD was the main goal of biofumigation studies, but results on disease control still vary as external factors like differences in temperature, precipitation and solar radiation as well as tissue disruption and soil water content can influence the effect of this method (Yim et al. 2016). Furthermore, the time needed for growing biofumigation plants results in lost production (Mazzola and Manici 2012). Therefore, application of *Brassica juncea* seed meal was deemed more efficient (Mazzola et al. 2007, 2009, 2015; Mazzola and Zhao 2010; El-Sharouny 2015). Additional alternative means for disinfection of ARD soils were proposed including the application of biochar (Wang et al. 2014), intact glucosinolates (Hanschen et al. 2015), arbuscular mycorrhiza as well as microbial (Guo et al. 2014; Gastol and Domagala-Swiatkiewicz 2015) and seaweed fertilizers (Wang et al. 2016), however, further tests have to be conducted. Other cropping practices, which may potentially mitigate the disease outcome, involve replanting in inter-rows (Kelderer et al. 2012), monitoring of soil urease activities (Sun et al. 2014), treatments for manipulation of rhizosphere microbial communities to decrease detrimental and increase beneficial microorganisms (Manici et al. 2013, 2015; Caputo et al. 2015; Yim et al. 2015),

and carbon source-dependent anaerobic soil disinfestation (Hewavitharana et al. 2014; Hewavitharana and Mazzola 2016).

While the counteractions mentioned above aimed to assure stable growth of established apple rootstocks, there is also the possibility of breeding more tolerant genotypes, which can better handle the adverse circumstances presented by ARD. Rootstock breeding (Volk et al. 2015) plus evaluation of ARD tolerance have recently been performed (Forge et al. 2016). Furthermore, phenotypical information referring to root turnover (Atucha et al. 2014) as well as root development (Emmett et al. 2014) have been linked to ARD tolerance mechanisms but the possible exploitation of genomics-assisted breeding relies on thorough understanding of ARD at the molecular level. It also depends on suitable traits associated with ARD and the development of closely linked molecular markers (Zhu et al. 2014).

So far, only few studies have been conducted in regard to the molecular responses of ARD-affected plants. It has been shown that phenolic compounds possibly function as antioxidant substances in response to ARD (Börner 1959; Emmett et al. 2014; Henfrey et al. 2015), especially, the flavonoid phloridzin was detected to a higher degree in exudates of ARD roots (Hofmann et al. 2009; Emmett et al. 2014). Correspondingly, first studies analyzed the transcriptomic response of apple seedlings to an infection by *Pythium ultimum*, one of the potential causal agents of ARD, which resulted in the upregulation of genes involved in secondary metabolism reactions next to differentially expressed genes in hormone metabolism amongst others (Shin et al. 2014, 2016; Zhu et al. 2014). Likewise, own previous results (Weiß et al. under revision) suggest the importance of secondary-metabolism-associated genes in the ARD response, which facilitates using such genes as molecular markers in genomics-assisted breeding.

Genes related to biphenyl and dibenzofuran phytoalexin biosynthesis could serve as such (negative) molecular markers, given that first results indicated the upregulation of biphenyl synthases, *O*-methyltransferases and biphenyl-4-hydroxylases in ARD roots after 7 days of cultivation in ARD soil (Weiß et al. under revision). The biosynthesis of these antimicrobial defense compounds was characterized using *Erwinia amylovora*-infected apple shoots and elicitor-treated *Sorbus aucuparia* cell cultures (Chizzali et al. 2012a, b, 2013; Khalil et al. 2015; Sircar et al. 2015). So far, ten biphenyls and 17 dibenzofurans were identified in Rosaceae, especially Malinae, species including *Malus*, *Pyrus* and *Sorbus* (Chizzali and Beerhues 2012; Khalil et al. 2013, 2015). Detection of these phytoalexins in roots is reported here for the first time.

Possible candidates for molecular markers have to be stably expressed in ARD-affected plants even when faced with ARD soils of different locations, i.e. with different properties and cropping history. Furthermore, candidates should show a strong expression for easy detection via RT-qPCR techniques. For understanding their function, it is also interesting to monitor the expression of candidates over time to obtain a better understanding of molecular responses in ARD challenged plants. Therefore, in this study, we aimed to investigate defense-related genes that were identified to

be significantly differentially expressed in ARD soil compared to disinfected ARD soil when using a RNA sequencing approach (Weiß et al. under revision) towards their time dependent response in ARD soils of different origin. We hypothesize that genes involved in ARD response are conserved regarding expression among ARD soils of different origin and can be distinguished from those being soil-specifically expressed. This study also aimed to contribute knowledge to defense reactions in roots of woody plants as much less information is available than for biotic stressors affecting aboveground organs of herbaceous species. The ARD vulnerable genotype 'M26'was employed due to its suitability as a reliable indicator test plant to detect ARD in soils (Yim et al. 2013).

Materials and methods

Soil origin and disinfection

Soil at a depth of 0-25 cm was taken from three field plots in a tree nursery in the area of Pinneberg, Schleswig-Holstein, Germany (53° 41' 58.51'' N, 9° 41' 34.12'' E) in September 2014. On this site (later on referred to as soil K), rose rootstock plants were grown from 1980 to 2011, with crop rotation with *Tagetes* starting from 2002 and annual replanting of the apple rootstock 'M4' started in 2012 (Yim et al. 2015, 2016). Amounts of 18-20 L of homogenized soil were packed in autoclavable bags (Sarstedt, Nümbrecht, Germany) and 175 L soil of the 350 L totally obtained were disinfected via γ-irradiation (BGS – Beta Gamma Service, Wiehl, Germany) at a minimum dose of 10 kGy, by which actinomycetes, fungi and invertebrates are eliminated (McNamara et al. 2003). Afterwards, the soil bags were stored at 4 °C for 10 days until one day before the start of the experiment.

Plant cultivation and harvest

In vitro propagation and rooting of the highly ARD susceptible apple rootstock 'M26' (Yim et al. 2013, 2015) was performed according to Weiß et al. (under revision). Acclimatization of rooted plants lasted three and a half weeks in the greenhouse. In December 2014, acclimatized plants were potted into 1 L pots containing either γ-irradiated replant soil (γARD) or untreated replant soil (ARD). The slow release fertilizer Osmocote Exact Standard 3-4 M (16-9-12+2MgO+TE, http://www.scottsprofessional.com) was added to the soils at a concentration of 2 g L^{-1} before potting of plants. Plants were grown under the following conditions: 19.33 °C ± 1.01 °C, 57.73 % ± 6.18 % relative humidity and a 16 h photoperiod with additional light (if solar radiation fell below 25 klx, provided by SONT Philips Master Agro 400W). Irrigation was carried out by hand on a daily basis as required. Weekly spraying against thrips and spider mites was performed as plant protection according to horticultural practice. In total 350 plants were used for the biotest, 175 plants each for γARD and ARD variants. Each soil variant included five different time points for harvest after 3, 7, 10, 14 and 56 days (end of experiment). For each time point, the shoot lengths of 35 plants were recorded. At the time points 3, 7, 10 and 14 days, root material of 25 of these plants was harvested for RNA isolation and subsequent gene expression analyses. Root material of the remaining 10 plants was used for the

analysis of phytoalexins. All plants cultivated for 56 days were used for weekly measuring shoot length.

For RNA isolation at days 0 (acclimatized plants before potting), 3, 7, 10 and 14, the whole root system was removed from the individual plants, rinsed with water, placed into 2 ml tubes (Sarstedt, Nümbrecht, Germany) and frozen in liquid nitrogen. Samples were stored at -80 °C until homogenization of plant material. Root material for the analysis of phytoalexins was treated similarly, with the exception that root material after washing was freeze-dried for 3 days (Christ ALPHA 1-4 LSC, Osterode, Germany) and stored at -20 °C until homogenization of plant material.

RNA extraction and first strand cDNA synthesis

Root materials of five plants were combined to form a biological pool (= biological replicate) and a total of five pools were generated for each time point and soil variant. The pools were homogenized as described by Weiß et al. (under revision) and RNA extraction from 100 mg homogenized root material was performed using InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) according to the manufacturer's instructions for phenol containing plants. RNA yield and quality were assessed by a spectrophotometer (Nanodrop 2000c, Peqlab, Erlangen, Germany). Subsequent genomic DNA removal was achieved by in-solution DNA digestion using 1 µg of total RNA and DNase I (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Afterwards, random hexamer primers were used in first strand cDNA synthesis to reversely transcribe total RNA, employing the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Aliquoted cDNA samples were stored at -80 °C for proceeding RT-qPCR experiments.

RT-qPCR gene expression analyses

Candidate and reference genes (*EF1a, EF1b* and *TUBB* according to expression stability; Supplementary Table S2) found by Weiß et al. (under revision) were analyzed with a real-time PCR cycler (CFX Connect™, Bio-Rad, Hercules, CA, USA). Primer combinations (200 nM for each forward/reverse primer), iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 1:10 cDNA dilution were mixed and data was recorded with the Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA). The protocol for RT-qPCRs was as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The program was ended after a melt curve analysis from 65 °C to 95 °C with an increment of 0.5 °C for 5 s at each step. Gene expression was measured for five biological replicates and two technical replicates per soil variant and time point. Normalized expression was calculated according to Pfaffl (2001).

Extraction and analysis of phytoalexins

By adding steel beads (ø 1.5 cm) to freeze-dried samples, root material was homogenized using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany). Afterwards, homogenized powder from

each individual variant (γARD, ARD) and time point (0, 3, 7, 10 and 14 days) was combined to obtain enough material for the analysis of phytoalexins. Phytoalexins were extracted from two technical replicates according to Chizzali et al. (2012b) and analyzed by GC-MS (Hüttner et al. 2010).

Statistical analyses

Using the statistics program R 3.3.2 (R Development Core Team 2008), statistical analyses were performed with $\log_2$ transformed data. Means of measured or calculated parameters between soil variants (γARD, ARD) were compared using the Welch Two Sample t-Test (Welch 1947) at a *P*-value level of 0.05. Different time points within one soil variant were compared using a Tukey multiple comparisons test adjusted for heterogeneous variances if needed (Tukey 1949; Herberich et al. 2010) at a *P*-value level of 0.05. For the test of correlation between gene expression data obtained at day 7 of this study and data from Weiß et al. (under revision), the Pearson product-moment correlation coefficient (Pearson 1895) was calculated in Microsoft Excel 2010.

For the expression data over time, the connection between the start point of the experiment and day 3 is indicated in the graphs by a dotted line, because the plant material at day 0 represented freshly uprooted acclimatized plantlets, which can only be partly compared to the freshly potted plantlets at day 3.

Results

ARD soil had a negative effect on the shoot and root growth of 'M26'

Plants of the apple rootstock 'M26' were negatively affected in their shoot growth over the course of the experiment when grown in ARD soil (Figure 1). After 7 days, plants grown in γARD soil showed significantly higher shoot length with 5.7 ± 1.1 cm compared to their counterparts cultivated in ARD soil with 5 ± 1.1 cm (Figure 1a). The differences between the two variants got more pronounced with increasing time, resulting in mean shoot lengths of 19.9 ± 5.3 cm and 7.9 ± 2.7 cm for γARD and ARD, respectively, at the end of the experiment, which indicates a 2.5-fold increase in shoot length in γARD plants. The habitus of the plants reflected these observations (Figure 1b). After 10 days of cultivation, the root system was also affected in overall growth and it showed darker coloration. Strikingly, shoot growth stagnated in both variants between days 10 and 21. Thereafter, only γARD plants showed massive shoot growth.

Genes involved in ARD response demonstrated both conservation and soil-specific behavior

In a previous study (Weiß et al. under revision), several genes associated with biotic stress response were found to be affected by ARD. To obtain more detailed information of the expression of these genes over time, the present study reports a kinetic experiment, employing soil from a different tree nursery with different properties and cropping history. For RT-qPCR experiments, the reference genes used were elongation factor 1-alpha (*EF1a),* elongation factor 1-beta 2-like (*EF1b)* and tubulin beta

chain (*TUBB)*. The data from Weiß et al. (under revision, hereafter referred to as soil A) were re-calculated based on the three reference genes used in this study to compare expression levels with results obtained in this study at day 7. Ratios between expression levels in ARD and γARD soils were revealed to be comparable for the majority of genes at least in tendency (Table 1) and they showed a positive relationship (Supplementary Figure S1). However, genes involved in phytoalexin biosynthesis were even more affected in soil K, as emphasized by a higher upregulation in ARD samples compared to soil A. For the caffeic acid 3-*O*-methyltransferase-like gene (*OMTb*), the biphenyl hydroxylase genes (*B4Ha* and *B4Hb*) and especially the biphenyl synthase genes (*BIS2* to *BIS4*), up to six times higher upregulation in ARD samples was observed in soil K compared to soil A, although the expression levels of γARD samples were almost equal in both soils (Supplementary Table S3). Likewise, the ethylene-responsive transcription factor 1B-like gene (*ERF1B*) revealed an even stronger upregulation in soil K with 12.03 higher expression in ARD samples compared to only 3.29 upregulation in soil A. Two additional noteworthy genes include the ethylene-responsive transcription factor RAP2-11-like (*ERF RAP2.11*) and the GATA zinc finger domain-containing protein 10-like (*GATAD10*). *ERF RAP2.11* was 12.93-fold downregulated in ARD samples in soil A, whereas this regulation was diminished in soil K to merely 2.07-fold. *GATAD10* displayed a similar behavior, showcasing a 15.06-fold upregulation in ARD samples in soil A but only 2.22-fold upregulation in soil K. Nevertheless, it has to be noted that the expression levels of these three signaling genes, albeit similar, were very low (Supplementary Table S3). Notwithstanding that 20 of the 27 analyzed genes displayed similar fold-change ratios in tendency for both soils, some genes were soil-specifically expressed in the roots of 'M26', such as the plant defense gene ankyrin-1-like (*ANK1*) being upregulated only 1.9-fold in soil K. Furthermore, the heat shock protein genes *HSP17.1, HSP17.3* and *HSP18.5* were only downregulated in ARD samples of soil A but either not regulated (*HSP17.1* and *HSP18.5*) or slightly upregulated (*HSP17.3*) in soil K. In the case of these three genes, the expression in γARD samples of soil A was also higher compared to soil K (Supplementary Table S3).

Plant defense genes were only slightly affected by ARD

The plant defense genes for ankyrin-1-like (*ANK1),* acidic endochitinase-like (*CHIA),* disease resistance protein At4g27190-like *(DRP)* and thaumatin-like protein 1a (*TL1)* exhibited diverse expression behavior (Figure 2). Whereas all genes showed a decrease in expression in both variants from the start of the experiment to day 3, the decrease was stronger in tendency for *ANK1*, *CHIA* and *TL1* in γARD samples. With the exception of *ANK1* and *DRP* (Figure 2a, c), expression of the other genes in γARD samples was more or less constant for the remaining time points. For *ANK1*, the expression levels in ARD soil were significantly higher at days 3, 7 and 14 compared to γARD samples. *CHIA* and *TL1* revealed an increase in gene expression over time in ARD samples at least in tendency but stable expression over time was observed for γARD samples (Figure 2b, d). In addition, *CHIA* showed significant differences between soil variants starting from day 3, whereas *TL1* was

significantly affected after 7 days of cultivation. Overall, significant upregulations in ARD samples were recorded, which ranged from two to three times for *ANK1* and *CHIA*, respectively, and up to nine times for *TL1*. *ANK1* and *CHIA* were expressed at the highest levels among the plant defense genes, followed by *TL1* and *DRP*, which were expressed lowest.

The selected genes with regulatory functions were negligibly expressed in ARD variants

The expression patterns of genes with regulatory functions also differed (Figure 3). While *HSP17.1, HSP18.5* and the F-box/kelch-repeat protein At4g12810 (*KFB*) and pectinesterase/pectinesterase inhibitor 41 (*PME41*) genes were not significantly influenced in their expression by ARD, *HSP 17.3* and the senescence-associated carboxylesterase 101-like gene (*SAG101*) were more highly expressed in ARD than in γARD samples, although at most time points at only slightly higher levels (Figure 3). Over time, expression of most genes declined during the first 3 days and thereafter did not show drastic changes, except for *PME41*, whose expression increased until day 3 and decreased thereafter (Figure 3e).

Signaling genes indicated diverse expression patterns

ERF RAP2.11, *ERF1B*, *GATAD10* and the genes for 1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (*ACO1*), dof zinc finger protein DOF3.5-like (*DOF3.5*), gibberellin-regulated protein 1-like (*GASA1*), LRR receptor-like serine/threonine-protein kinase MRH1 (*MRH1*), NAC transcription factor 25-like (*NAC25*), wound-induced protein 1-like (*WIN1*) and zinc finger protein ZAT12 (*ZAT12*), which are classified as genes involved in signaling, showed differing expression patterns and low expression levels (Figure 4). *ACO1*, *ERF RAP2.11*, *GASA1*, *MRH1* and *WIN1* exhibited significantly higher expression in γARD samples, at least at one time point during the experiment (Figure 4a, c, e, g, i). Expression of these genes in ARD samples stayed on a consistent level, except for *WIN1* with higher expression at day 14 compared to the other time points. In γARD samples, the genes were stably expressed until day 7 after an initial decrease from the start of the experiment to day 3, except for *ACO1*, whose expression further decreased at day 7 and then did not differ for the remaining time points (Figure 4a). *ERF RAP2.11*, *GASA1*, *MRH1* and *WIN1* increased in expression after day 7, showing maximum expression at day 10 with significant differences compared to ARD samples. They stayed on this level also at day 14. Expression of *ERF1B*, *GATAD10* and *ZAT12* was significantly higher in ARD samples for all time points with the exception of day 3 for *GATAD10* (Figure 4d, f, j).

Phytoalexin biosynthesis genes revealed a strong and early response to ARD

The phytoalexin biosynthesis genes *BIS2*, *BIS3*, *BIS4*, *OMTb*, *B4Ha* and *B4Hb* showed similar expression profiles over time (Figure 5), although at different expression levels. These genes were significantly upregulated in ARD samples already after 3 days and showed an increase in expression compared to the start of the experiment. Expression did not significantly increase at day 7 but after

10 days a further rise in expression was measured for these genes at least in tendency. Afterwards, expression levels stayed constant at day 14. Expression of these genes in γARD samples consistently remained on a lower level with a slight decrease measured from the start of the experiment to day 3. Only the caffeic acid 3-*O*-methyltransferase-like gene (*OMTa*) did not follow this expression pattern (Figure 5d), but displayed hardly any differences between soil variants and time points. Regarding the different expression levels, *BIS2* and *BIS3* were expressed highest in ARD samples with peak levels reaching values of normalized expression of 26.48 ± 7.33 (21.18 times upregulated) and 57.27 ± 14.91 (11.36 times upregulated) at day 10, respectively (Figure 5a, b). In ARD samples at day 10, the phytoalexin biosynthesis genes *OMTb*, *B4Ha* and *B4Hb* showed similar maximal normalized expression values of 14.1 ± 4.26 (3.64 times upregulated), 7.66 ± 2.72 (6.47 times upregulated) and 10.42 ± 3.09 (6.71 times upregulated), respectively (Figure 5e-g). In contrast, *BIS4* and *OMTa* were expressed at day 10 at lower levels with maximal normalized expression values of 2.53 ± 0.72 (26.23 times upregulated) and 3.05 ± 0.39, respectively (Figure 5c, d).

High levels of phytoalexins were detected in ARD samples

Corresponding to the increased expression levels of phytoalexin biosynthesis genes, the total phytoalexin content in ARD samples was found to be consistently higher than in γARD samples (Figure 6). In ARD samples, the total content over time showed a trend upwards, reaching 1.7 ± 0.19 mg/g dry matter after 14 days. In contrast, contents in γARD samples stayed on the same level at around 0.2 mg/g dry matter over time after an initial decrease from the start of the experiment. Individual phytoalexin compounds revealed the same tendency of being present at higher concentrations in ARD samples (Table 2). 3-Hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2-hydroxy-4-methoxydibenzofuran, 2'-hydroxyaucuparin and noreriobofuran were identified in ARD samples. The compound 2-hydroxy-4-methoxydibenzofuran was found over all time points in γARD samples but it was detected at higher concentrations in ARD samples. The other compounds were either not detected or identified at low concentrations in γARD samples. 3-Hydroxy-5-methoxybiphenyl and aucuparin were exclusively found in ARD samples. Noraucuparin was also detected in γARD samples at day 3 but at a 13 times lower concentration than in ARD samples. Figure 7 summarizes the kinetics on the accumulation of the detected phytoalexins and the expression of the corresponding genes in a proposed biosynthetic pathway of phytoalexins found in 'M26'.

Discussion

ARD rapidly affected the growth habitus of 'M26' plants

Molecular responses of apple plants affected by ARD are not yet well understood but progress has recently been made in uncovering ARD in the plant on the molecular level (Shin et al. 2014, 2016; Zhu et al. 2014; Henfrey et al. 2015; Weiß et al. under revision). The present study aimed to gather more data to better comprehend ARD responses on the molecular level and to contribute knowledge to

defense reactions in roots of woody plants, for which much less information is available than for biotic stressors affecting aboveground organs of herbaceous species. Based on the results of a preceding study (Weiß et al. under revision), selected ARD-affected candidate genes were investigated over time, using a soil of a different tree nursery and employing a time course expression analysis. The conserved expression of genes between different soils might indicate their importance in ARD. Early-reacting genes may be employed for early monitoring in order to estimate the ARD severity of soils.

In this study, shoot growth of the ARD susceptible apple rootstock 'M26' was affected already after 7 days of cultivation in ARD soil, thus even earlier than in previous investigations (Yim et al. 2015, Weiß et al. under revision). Since the cited reports also focus on the ARD prone apple rootstock 'M26', the soil used in our study appears to be more severely affected by ARD. This is probably due to differing cropping history (apple rootstock plants on soil A were repeatedly grown until 2009, followed by *Prunus domestica* in 2010, *Cydonia oblonga* in 2011 and annual replanting of the apple rootstock 'M4' since 2012; Yim et al. 2015, 2016) as well as different soil properties (Supplementary Table S1), resulting in different microbial communities in these two soils (Yim et al. 2015).

While γ-irradiation of the soil majorly improved shoot growth, ARD plants barely showed an increase in the measured parameter over time, indicating the connotation of ARD with detrimental microorganisms. Yim et al. (2015) discussed the greater abundance of genera with possibly advantageous characteristics in disinfected soil, leading to improved growth and thereby hinting at soil microbial community shifts. Plants grown in γARD soil showed a 4.6-fold increase in shoot length whereas ARD plants revealed only a 1.8-fold increase, resulting in a 2.5 times shoot length difference between γARD and ARD plants. Growing 'M26' plants on ARD soil from a different tree nursery resulted in a 1.8-fold shoot length difference (Weiß et al. under revision). Therefore, the soil used in this study appeared to be more heavily affected by microorganisms leading to ARD, and γ-irradiation was able to effectively disinfect ARD soil from detrimental microorganisms. Beside shoots, roots of ARD-exposed plants were affected as well. After 10 days, roots cultivated in ARD soil demonstrated diminished overall growth combined with darker coloration. Root discoloration of ARD-challenged plants was frequently reported previously (Tewoldemedhin et al. 2011a; Mazzola and Manici 2012; Yim et al. 2013; Atucha et al. 2014; Shin et al. 2014; Henfrey et al. 2015; Weiß et al. under revision). Stronger lignification of ARD-affected roots, indicating oxidation of phenolic compounds, was found in 'M26' (Yim et al. 2013). It may suggest that higher phenolic concentrations in the root system were responsible for the color of roots in ARD samples. Phenolic compounds may play a role as antioxidant substances in defense to ARD (Emmett et al. 2014; Henfrey et al. 2015). Especially, the flavonoid phloridzin was found to a higher extent in root exudates of ARD plants (Hofmann et al. 2009; Emmett et al. 2014). Transcriptomic data obtained for *P. ultimum*-infected apple roots and ARD-affected 'M26' roots also emphasize the involvement of phenolic metabolism, such as flavonoid and

phenylpropanoid pathways, in ARD due to the upregulation of associated genes (Shin et al. 2016; Weiß et al under revision).

Diverse expression of biotic stress response genes in 'M26' advocates suppressed defense reactions

Genes functioning in ROS and antioxidant metabolism as well as kinase signaling have recently been reported to be differentially expressed after infection of apple seedlings with *P. ultimum* (Shin et al. 2016). The authors concluded that ROS scavenging systems such as ascorbate and glutathione may play a critical role in apple root tissues with high levels of ROS due to *P. ultimum* infection. Interestingly, genes associated to the ascorbate and glutathione redox systems via MapMan were also more often but only minimally upregulated in ARD samples cultivated in soil A (Weiß et al. under revision), pointing to the possibility of a non-sufficient ROS scavenging system in the ARD susceptible rootstock 'M26', leading to root cell death.

ACO1 plays an essential role in the biosynthesis of ethylene, which is often reported to act in defense reactions towards biotic stress (Glazebrook 2005; Broekaert et al. 2006). Here, we could show that *ACO1* was consistently expressed at lower levels in ARD samples, indicating either a diminished or a suppressed role in defense towards ARD. The same was true for *ERF RAP2.11* but not *ERF1B*, which showed an upregulation in ARD samples. In contrast, *ACO1* and ethylene responsive transcription factors were upregulated in the study of Shin et al. (2014), which deals with the infection of apple plants with one of the causal agents of ARD, *P. ultimum*. Therefore, in our study, ethylene signaling may have been suppressed in ARD samples. However, activation of downstream defense related genes such as *CHIA* and *TL1* was observed, despite the obvious lack of their reported effects in defense towards biotic stress (Liu et al. 2010; Shin et al. 2014). For a complete picture, more genes involved in ethylene biosynthesis and signaling need to be investigated.

While the signaling genes *DOF3.5* and *NAC25* showed hardly any response to ARD, contrary to previous results indicating a soil-specific expression of these genes (Weiß et al. under revision), the transcription factors *GATAD10* and *ZAT12* demonstrated higher expression over time in ARD samples compared to γARD samples. The function of *ZAT12* in biotic stress response is associated to ROS signaling (Davletova et al. 2005), further highlighting the importance of ROS scavenging, as mentioned earlier. The disturbed defense mechanisms of ARD susceptible rootstock 'M26' was also shown by the downregulation over time of *GASA1*, *MRH1* and *WIN1*, which are responsible for regulating DELLA proteins and thereby modulate the balance of salicylic acid and jasmonic acid signaling in plant immunity (Zhu et al. 2005; De Bruyne et al. 2014), disease resistance signal cascades (Afzal et al. 2008) and wounding-induced signaling, which in turn lead to phytohormone and ROS signaling (León et al. 2001). Also, genes with regulatory function, tested due to their role in maintaining a balanced metabolism even when faced with the biotic stress of ARD, gave no clear indication on why 'M26' showed typical defense reaction towards biotic stress with no adequate visible response.

Compromised defense reactions in ARD-affected 'M26' roots suggest phytoalexin toxicity

The observation that plants grown on ARD soil used in this study (soil K) were more severely affected by ARD than those in the previous study (soil A) may also be correlated to the higher upregulation of phytoalexin biosynthesis genes at day 7 in this study. While soil A revealed 3.7 to 4.1 times upregulation of *BIS2*, *BIS3* and *BIS4* in ARD roots, soil K resulted in upregulation of these genes in ARD roots up to 24 times at day 7. Meanwhile, γARD samples demonstrated similar expression levels in both soils. In addition, *B4Ha* and *B4Hb* were two times more highly expressed in ARD samples of soil K than in soil A. In phytoalexin biosynthesis, the aforementioned genes are involved in the production of biphenyls and dibenzofurans (Figure 7; Khalil et al. 2015). To deal with biotic stress, reported to be the main cause of ARD incidence (Mazzola and Manici 2012), plants have the opportunity to produce phytoalexins (Ahuja et al. 2012). In apple, biphenyls and dibenzofurans were found in higher concentrations in the transition zone of *Erwinia amylovora*-infected shoots as a result of higher biphenyl synthase gene expression, especially *BIS3* (Chizzali et al. 2012a, b, 2013). In our study, *BIS3* was likewise the gene with the highest expression level whereas *BIS2* and *BIS4* showed the highest upregulation of monitored candidate genes. This is in accordance with a previous study, in which biphenyl synthase genes were also expressed at the highest level in ARD samples (Weiß et al. under revision). The importance of these genes was also indicated by infection of apple roots with *P. ultimum*, one of the potential causal agents of ARD, given the upregulation of *BIS4* (annotated as chalcone synthase) 24 hours post inoculation (Shin et al. 2016). The high expression of phytoalexin biosynthesis genes under ARD conditions further supports the predominant role of fungi in causing ARD (Manici et al. 2013; Franke-Whittle et al. 2015). Phytoalexins cause disturbance of membrane integrity (Rogers et al. 1996), constraint of conidial germination and germ-tube prolongation (Sellam et al. 2007) as well as apoptotic-like programmed cell death of fungi (Shlezinger et al. 2011). In healthy plant materials phytoalexins are commonly not present (Morrissey and Osbourn1999), which is also true for the biphenyls studied here (Figure 7). However, two dibenzofurans were detectable at day 0. Either the physiological state of the plants was perturbed upon planting, as supported by the decreasing levels of noreriobofuran in γARD samples, or the compounds may also function as constitutive phytoanticipins, which is supported by the relatively constant levels of 2-hydroxy-4-methoxydibenzofuran. Nevertheless, the important function of biphenyls and dibenzofurans as phytoalexins is indicated by the rapid and strong upregulation of the associated biosynthetic genes towards ARD, as shown for *BIS2*, *BIS3*, *BIS4*, *OMTb*, *B4Ha* and *B4Hb*, whose expression levels were increased already after 3 days of cultivation in ARD soil. *OMTa* was the only gene not to be upregulated after day 3, indicating that different isoforms of genes take over different roles in the ARD response.

In potato, *Vicia fabia* and *Phaseolus vulgaris*, host resistance was correlated to the level of phytoalexin accumulation but *Brassica* spp. and various cruciferous plants lacked this relationship and high phytoalexin concentrations potentially led to cytotoxicity (Rogers et al. 1996). Dixon et al. (1994)

also assumed that the high accumulation of phytoalexins is toxic to the plant. The phytoalexin phaseolin killed bean and beet cells, possibly due to the loss of tonoplast integrity, leading to the release of toxic plant metabolites and hydrolytic enzymes (Glazener and Van Etten 1978; Hargreaves 1980). One may hypothesize that the ARD sensitive rootstock 'M26' cannot exploit the phytoalexin defense mechanism. Rather, the observed response in roots of this genotype may have led to conditions, under which either sequestration or exudation of potentially toxic molecules resulted in killing of parts of the root system. This assumption might be supported by the early shoot length differences observed in this study between γARD and ARD plants after 7 days, correlated to the early and high expression of phytoalexin biosynthesis genes in ARD roots and the resulting elevated phytoalexin content compared to γARD samples. The finding that the expression of phytoalexin biosynthesis genes did not decrease over time but showed an additional peak after 10 days might be due to the fact that newly developing lateral roots continuously get in contact with ARD. This hypothesis is fostered by the observation of brown and non-viable roots or root parts in ARD soil. Due to the responsibility of roots to provide the plant with nutrients and water, shoot growth was negatively affected.

The biphenyl and dibenzofuran phytoalexins 3-hydroxy-5-methoxybiphenyl, aucuparin, noraucuparin, 2'-hydroxyaucuparin and noreriobofuran, with high contents in ARD roots, were previously detected in the transition zone of fire blight-infected shoots of 'Holsteiner Cox' (Chizzali et al. 2012a, b, 2013). Likewise, noraucuparin, aucuparin and 2'-hydroxyaucuparin were found in fire blight-infected shoots of 'Golden Delicious' (Sircar et al. 2015). The total content of biphenyls and dibenzofurans after 42 days amounted to 0.43 ± 0.08 mg/g dry matter (Chizzali et al. 2012a, b, 2013), although local concentrations within the transition zone may be much higher. In the present study, the total phytoalexin content added up to approximately 1.7 mg/g dry matter in ARD samples after 14 days, whereas γARD samples accumulated only around 0.2 mg/g dry matter. Rogers et al. (1996) demonstrated the cytotoxicity of the phytoalexin camalexin in *Arabidopsis* at concentrations of 0.5 mM and 2.5 mM, whereas phaseolin at 0.05 mM and 0.1 mM was able to kill bean and beet cells (Glazener and Van Etten 1978; Hargreaves 1980). In our study, individual phytoalexins reached peak concentrations of 1.9 mM (aucuparin, noraucuparin), hence, cytotoxicity of phytoalexins may play a role in ARD-affected 'M26' plants. More tolerant genotypes may possess the ability to have either a better control of the phytoalexin production, a different phytoalexin composition or a more potent detoxification system, as proposed by Henfrey et al. (2015). 3-Hydroxy-5-methoxybiphenyl, aucuparin and noraucuparin were almost exclusively found in ARD samples and may hence play a superior role in this regard.

Conclusions

The expression of most analyzed candidate genes was conserved in 'M26' after a 7-day-cultivation on ARD soils of different origin and cropping history, as highlighted by ratios comparable in tendency

between ARD and γARD. This supports the suitability of this genotype in testing soils for incidence of ARD, but information about gene expression also has to be related to soil microbial communities in ARD soils for full comprehension of the plant responses. Furthermore, this study emphasized the importance of phytoalexin biosynthesis genes and their role in ARD. Microorganisms inciting the biotic stress of ARD lead to accumulation of high amounts of phytoalexins in 'M26', whose potential cytotoxicity may evoke cell death, in combination with possibly deadly levels of ROS, and possible exudation into the soil may influence rhizosphere microbial communities affecting the ARD biome (Figure 8). Future studies will focus on uncovering the potential toxic effect of these compounds on the rootstock genotype 'M26' and the comparison of the present results with those obtained with other more tolerant genotypes. Also, the hypotheses of controlled phytoalexin biosynthesis, different phytoalexin compositions and more efficient detoxification systems in other genotypes have to be considered, as this information can be used in genomics-assisted breeding for the selection of more tolerant genotypes. Finally, the possibility of exudation of phytoalexins into the rhizosphere and the resulting effects of the defense compounds on the microbial communities in the rhizosphere require further investigations.

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3.2. Figures and tables

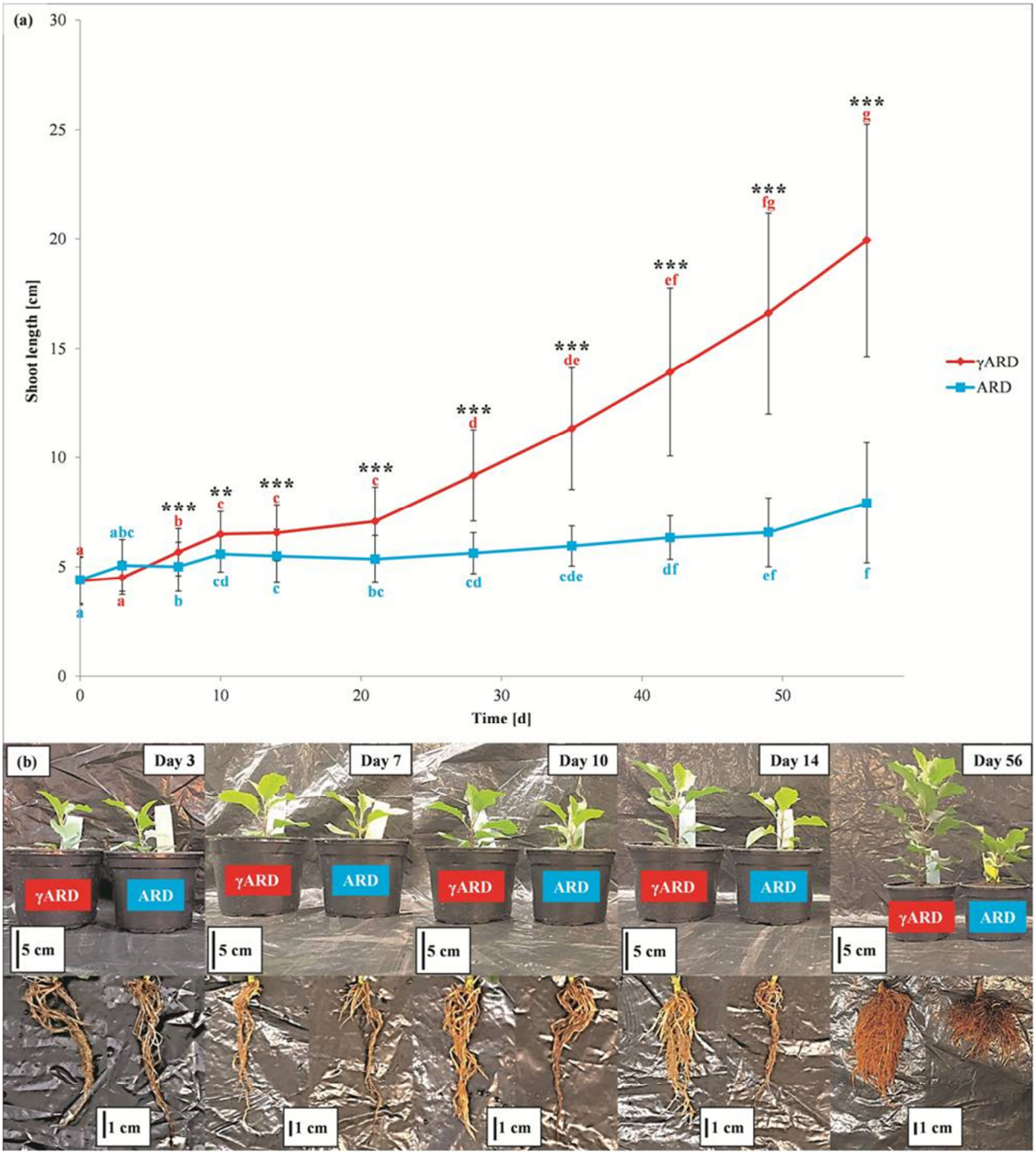


Figure 1. Response of 'M26' plants to γARD and ARD soils. (a) Shoot length development over time. Differences between treatments at one time point were tested using a Welch Two Sample t-test (means and standard deviations of $n_0 = 175$, $n_{3;10;21;28;35;42;49;56} = 35$, $n_7 = 140$, $n_{14} = 70$) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were marked with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD). (b) Overall habitus of plants cultivated in either γARD or ARD soils after 3, 7, 10, 14 and 56 days (end of experiment) with corresponding root systems.

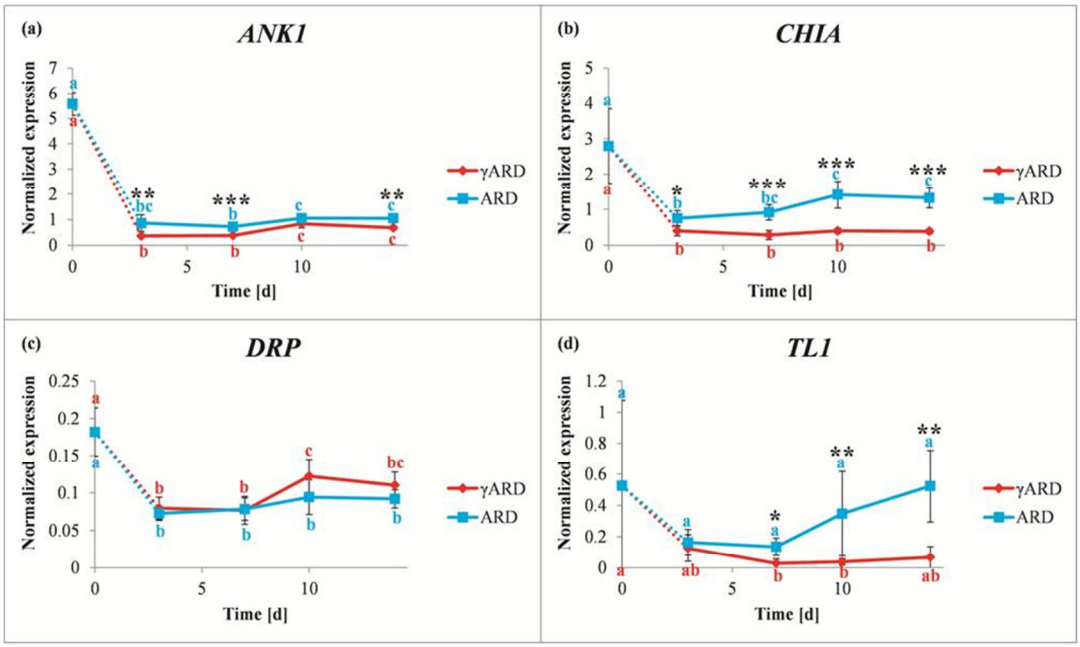


Figure 2. Expression of plant defense genes in γARD and ARD samples analyzed by RT-qPCR. Differences between treatments for *ANK1* (a), *CHIA* (b), *DRP* (c) and *TL1* (d) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were marked with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD).

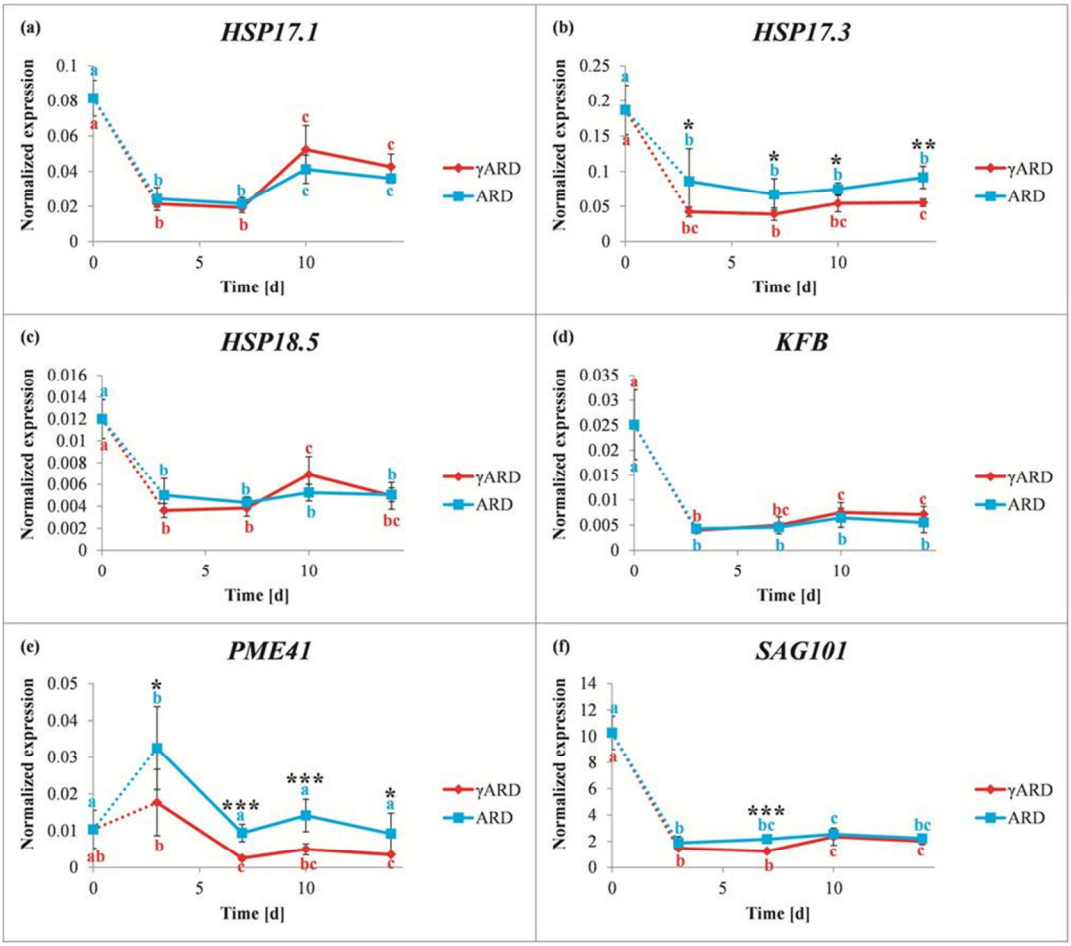


Figure 3. Expression of genes with regulatory functions in γARD and ARD samples analyzed by RT-qPCR. Differences between treatments for *HSP17.1* (a), *HSP17.3* (b), *HSP18.5* (c), *KFB* (d), *PME41* (e) and *SAG101* (f) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were marked with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD).

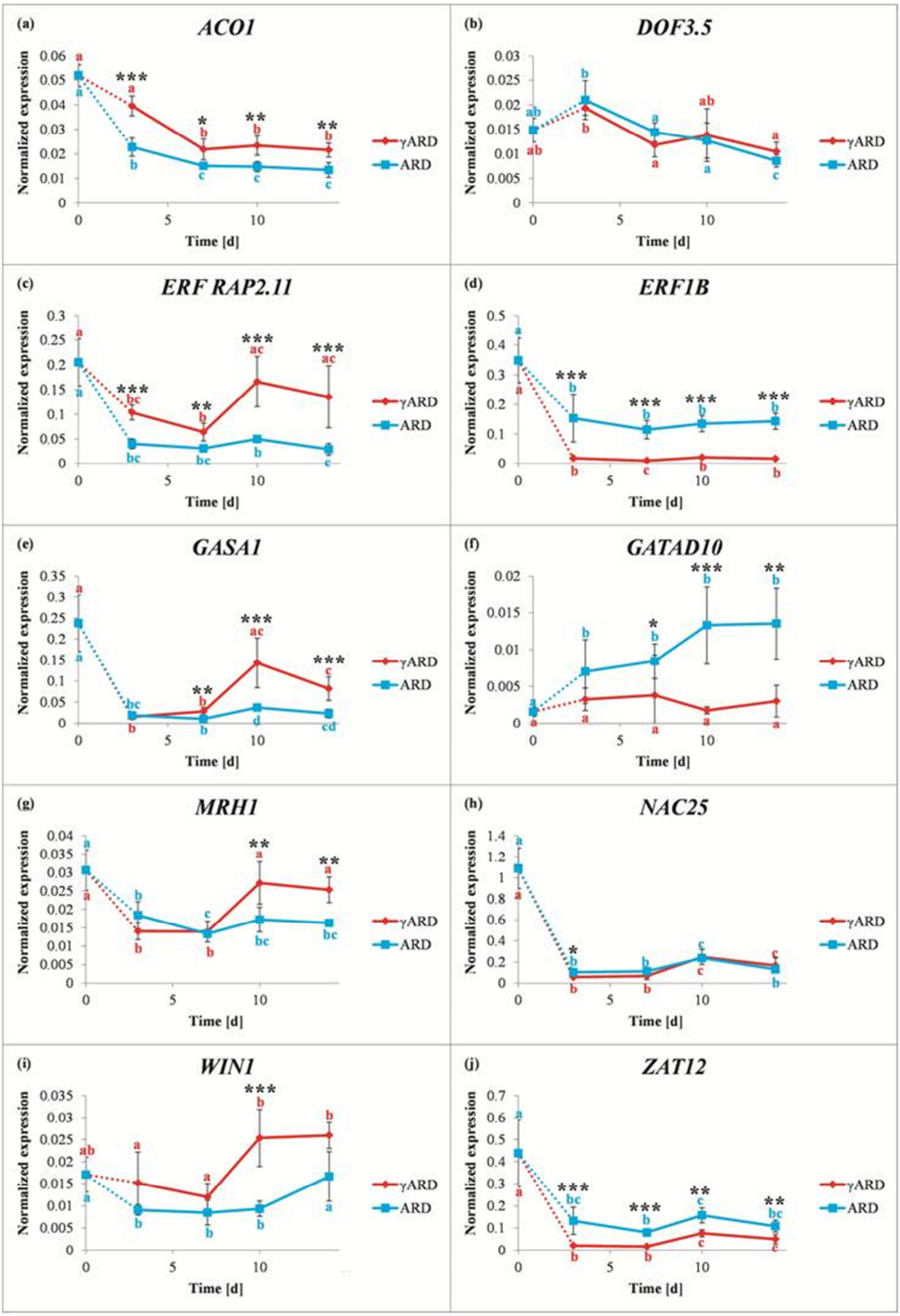


Figure 4. Expression of signaling genes in γARD and ARD samples analyzed by RT-qPCR. Differences between treatments for *ACO1* (a), *DOF3.5* (b), *ERF RAP2.11* (c), *ERF1B* (d), *GASA1* (e), *GATAD10* (f), *MRH1* (g) *NAC25* (h) *WIN1* (i) and *ZAT12* (j) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5, *GATAD10* day 3: n = 3) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were marked with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD).

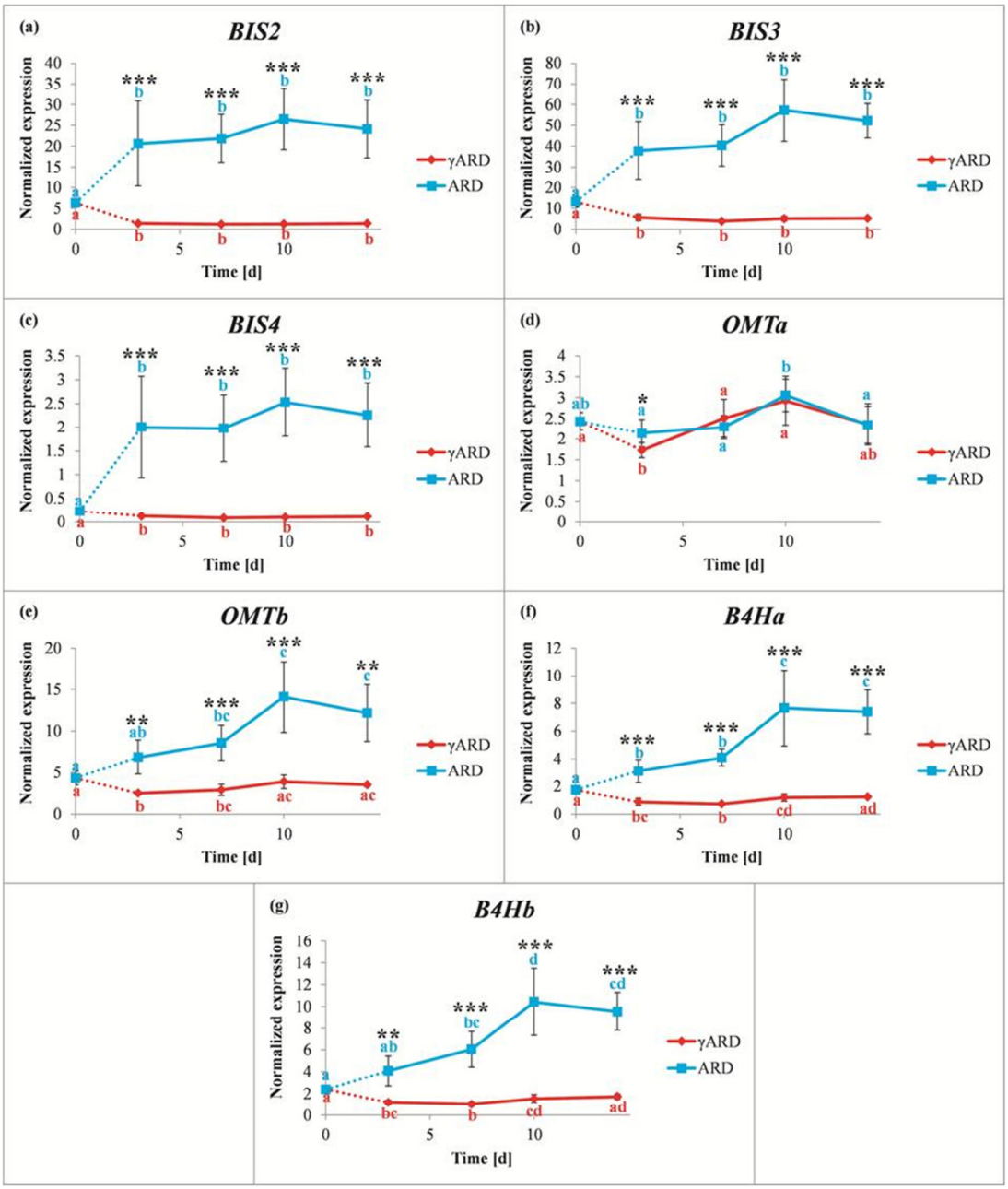


Figure 5. Expression of phytoalexin biosynthesis genes in γARD and ARD samples analyzed by RT-qPCR. Differences between treatments for *BIS2* (a), *BIS3* (b), *BIS4* (c), *OMTa* (d), *OMTb* (e), *B4Ha* (f) and *B4Hb* (g) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were marked with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD).

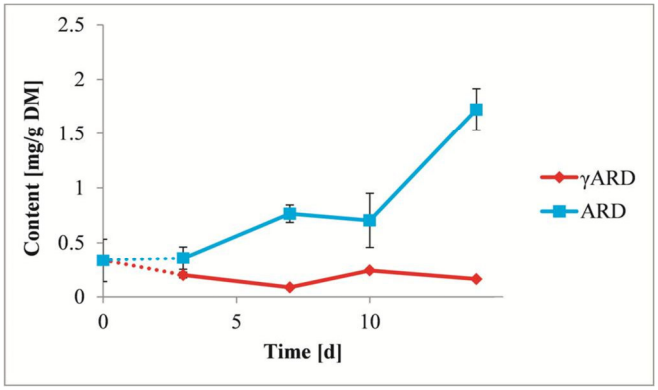


Figure 6. Total phytoalexin content in mg per g dry matter in γARD and ARD samples. Means and standard deviations of two technical replicates of one pooled biological replicate are depicted.

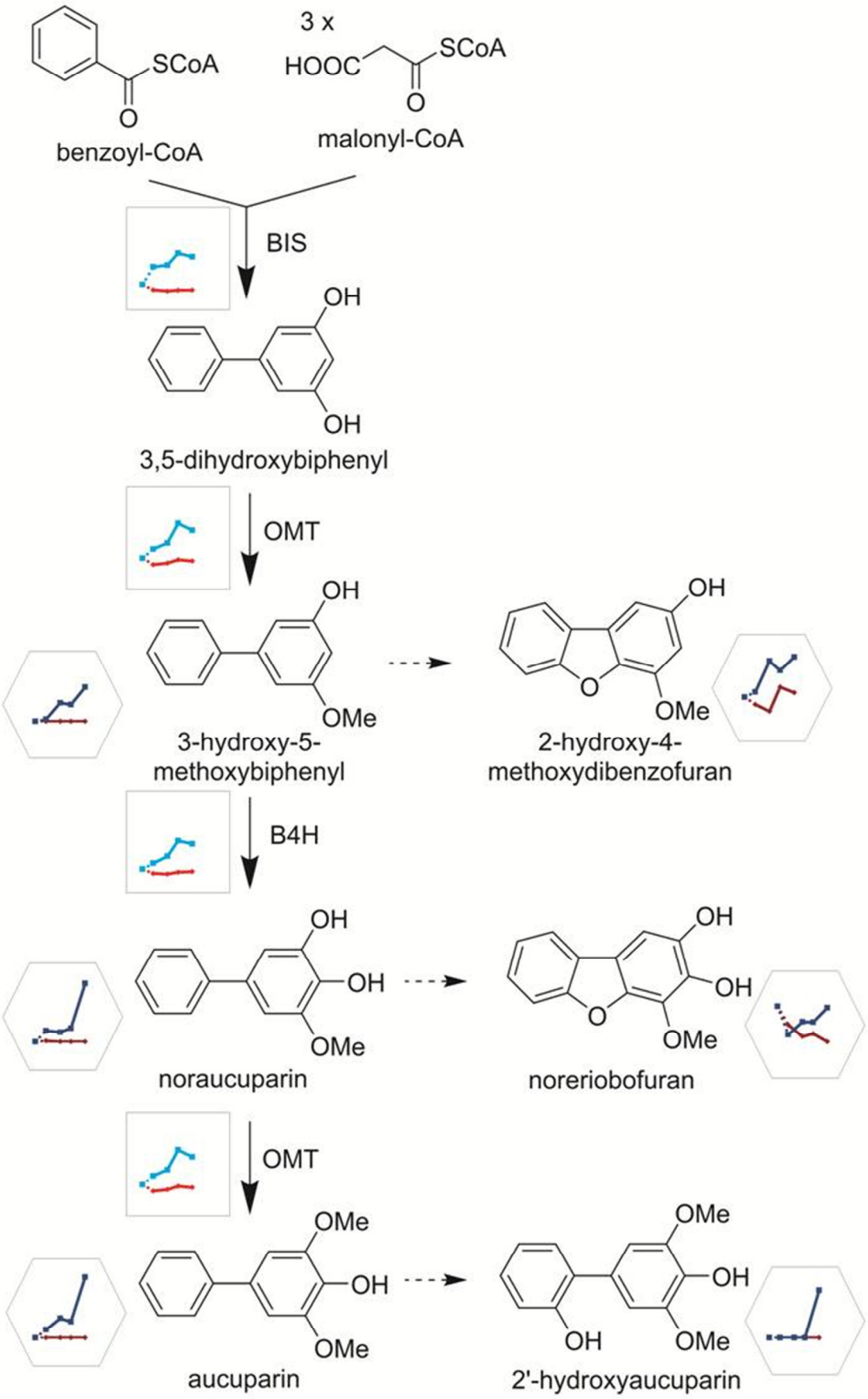


Figure 7. Proposed biosynthetic pathway of phytoalexins in 'M26'. Solid arrows represent identified enzymatic reactions whereas broken arrows mark unidentified enzymatic reactions. Small graphs represent changes in gene expression (square: red color for γARD, blue color for ARD) for corresponding enzymes (BIS, OMT, B4H) and in content of individual detected compounds (hexagon: dark red color for γARD, dark blue color for ARD).

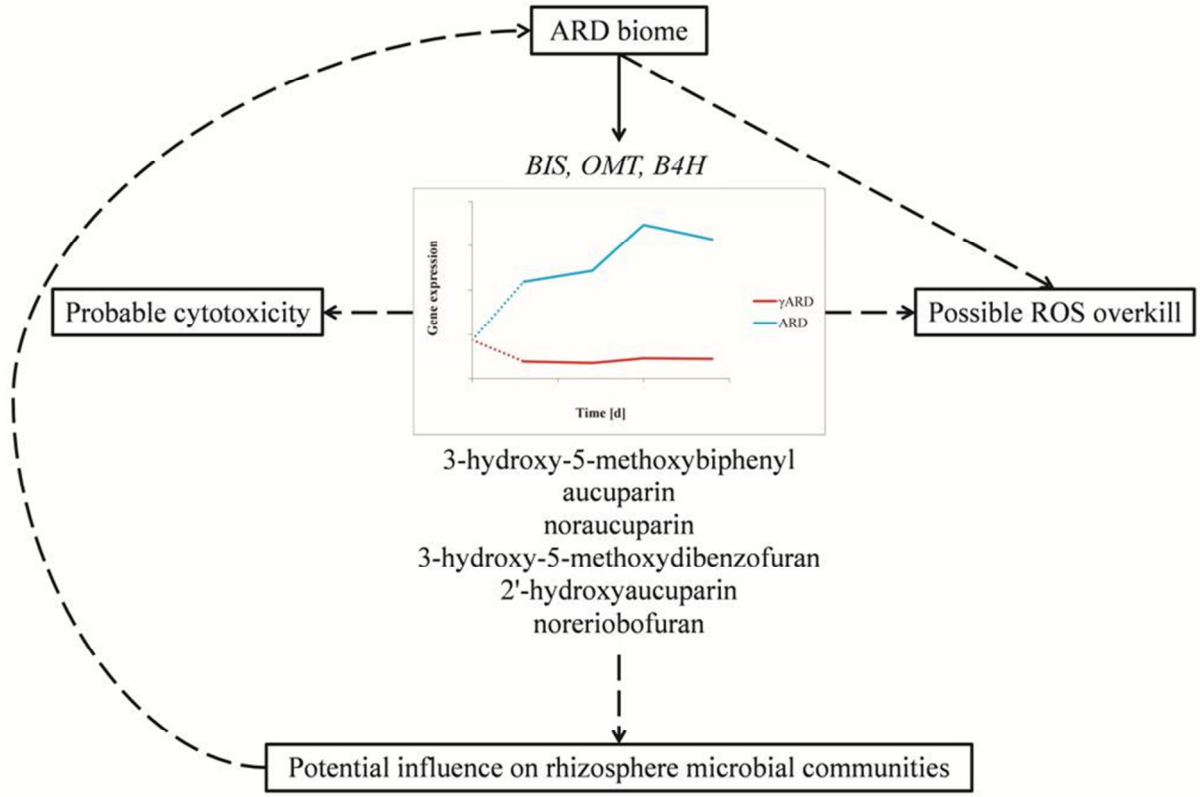


Figure 8. Supposed causal effects in the molecular response of ARD-affected 'M26' roots. The solid arrow represents the observed effect of ARD on phytoalexin biosynthesis whereas broken arrows suggest potential causes in ARD.

Table 1. Relative gene expression levels analyzed by RT-qPCR in roots cultivated in soils from different nurseries at day 7.*

Class	Gene	Gene name	Ratio soil A	Ratio soil K
Phytoalexin biosynthesis	***BIS2***	**biphenyl synthase 2 (MDP0000716308)**	**3.67***	**18.5****
Phytoalexin biosynthesis	***BIS3***	**biphenyl synthase 3 (MDP0000287919)**	**4.11****	**10.58****
Phytoalexin biosynthesis	***BIS4***	**biphenyl synthase 4 (MDP0000302905)**	**3.9***	**23.95****
Phytoalexin biosynthesis	*OMTa*	caffeic acid 3-O-methyltransferase-like (MDP0000456277)	1.27	-1.09
Phytoalexin biosynthesis	***OMTb***	**caffeic acid 3-O-methyltransferase-like (MDP0000745475)**	**2.05****	**2.96****
Phytoalexin biosynthesis	***B4Ha***	**cytochrome P450 CYP736A12-like (MDP0000205306)**	**2.78****	**5.62****
Phytoalexin biosynthesis	***B4Hb***	**cytochrome P450 CYP736A12-like (MDP0000152900)**	**2.61****	**5.83****
Plant defense	*ANK1*	ankyrin-1-like (MDP0000157404)	-1.08	1.9***
Plant defense	***CHIA***	**acidic endochitinase-like (MDP0000218691)**	**2.8*****	**3.21*****
Plant defense	*DRP*	disease resistance protein At4g27190-like (MDP0000463166)	-1.17	1.02
Plant defense	***TL1***	**thaumatin-like protein 1a (MDP0000552328)**	**4.32****	**4.88***
Regulatory function	*HSP17.1*	17.1 kDa class II heat shock protein-like (MDP0000700383)	-3.49***	1.11
Regulatory function	*HSP17.3*	17.3 kDa class II heat shock protein-like (MDP0000548065)	-3.37***	1.72*
Regulatory function	*HSP18.5*	18.5 kDa class I heat shock protein-like (MDP0000759666)	-3.9***	1.13
Regulatory function	*KFB*	F-box/kelch-repeat protein At4g12810 (MDP0000708634)	6.29***	-1.08
Regulatory function	***PME41***	**pectinesterase/pectinesterase inhibitor 41 (MDP0000798705)**	**2.39***	**3.82*****
Regulatory function	*SAG101*	senescence-associated carboxylesterase 101-like (MDP0000837935)	1.11	1.75***
Signaling	***ACO1***	**1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (MDP0000314499)**	**-2.35****	**-1.44***
Signaling	*DOF3.5*	dof zinc finger protein DOF3.5-like (MDP0000308863)	1.42*	1.22
Signaling	***ERF1B***	**ethylene-responsive transcription factor 1B-like (MDP0000127134)**	**3.29****	**12.03*****
Signaling	***ERF RAP2.11***	**ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)**	**-12.93*****	**-2.07****
Signaling	***GASA1***	**gibberellin-regulated protein 1-like (MDP0000140078)**	**-1.95****	**-2.66****
Signaling	***GATAD10***	**GATA zinc finger domain-containing protein 10-like (MDP0000922823)**	**15.06*****	**2.22***
Signaling	*MRH1*	LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	-1.37*	-1.04
Signaling	*NAC25*	NAC transcription factor 25-like (MDP0000842702)	1.95*	1.65
Signaling	*WIN1*	wound-induced protein 1-like (MDP0000792101)	-2.44***	-1.42
Signaling	***ZAT12***	**zinc finger protein ZAT12 (MDP0000595671)**	**4.14*****	**4.77*****

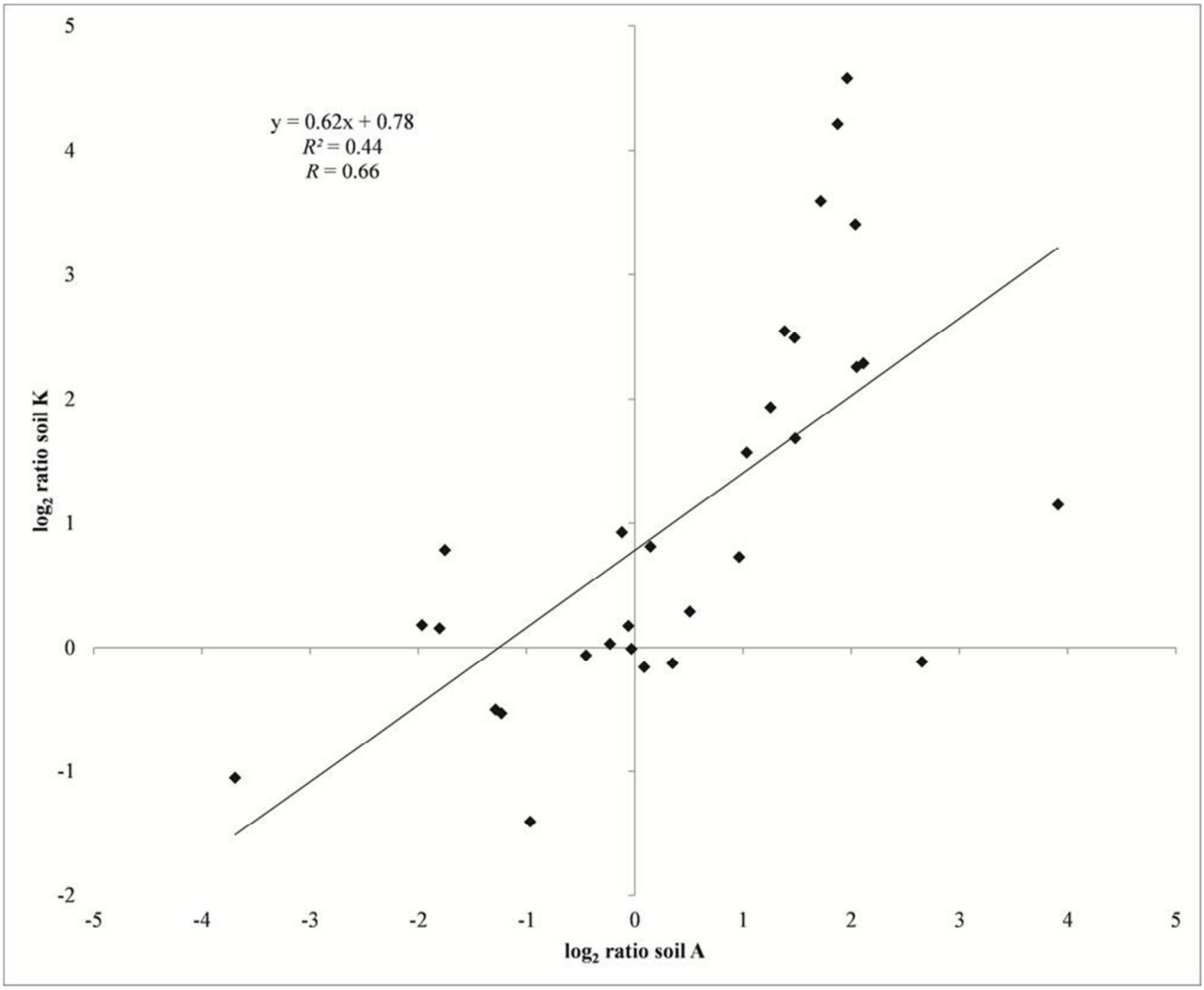
*For normalization, *EF1a, EF1b and TUBB* were used as reference genes. Ratios are represented by the quotient of ARD/γARD with values between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Data from Weiß et al. (under revision) was recalculated to obtain comparable expression values and the resulting ratios (Ratio soil A) are compared to ratios at day 7 from data obtained in this study (Ratio soil K). Differences between treatments were tested using a Welch Two Sample t-test (n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Genes are alphabetically ordered according to their class. Bold printing indicates consistent expression behavior between the two soils.

Table 2. Changes in phytoalexin contents (µg per g dry matter).*

Compound	**Day 0**	**Day 3**		**Day 7**		**Day 10**		**Day 14**	
		γARD	ARD	γARD	ARD	γARD	ARD	γARD	ARD
3-hydroxy-5-methoxybiphenyl	n. d.	n. d.	11.92 ± 16.86	n. d.	104.6 ± 11.6	n. d.	94.56 ± 45.69	n. d.	193.73 ± 13.55
aucuparin	n. d.	n. d.	58.13 ± 22.78	n. d.	137.97 ± 7.47	n. d.	114.41 ± 39.34	n. d.	426.95 ± 14.56
noraucuparin	n. d.	6.41 ± 9.07	79.6 ± 39.4	n. d.	68.04 ± 96.23	n. d.	96.58 ± 66.75	n. d.	410.54 ± 5.28
2-hydroxy-4-methoxydibenzofuran	137.33 ± 76.61	95.75 ± 13.37	166.16 ± 71.66	56.22 ± 7.52	341.82 ± 33.14	194.82 ± 10.53	287.76 ± 73.91	162.3 ± 7.1	364.82 ± 18.47
2'-hydroxyaucuparin	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	133.78 ± 126.84
norerioboфuran	199.31 ± 120.59	96.88 ± 3.79	40.77 ± 11.76	29.81 ± 4.19	113.16 ± 11.73	45.63 ± 7.61	111.2 ± 21.42	n. d.	192.14 ± 50.67

*Values indicate means and standard deviations of two technical replicates; n. d. not detectable.

3.3. Supplementary data



Supplementary Figure S1. Correlation of $\log_2$ ratios obtained for soil A and soil K at day 7. The $\log_2$ ratios of ARD/γARD are shown (n = 30). Pearson product-moment correlation coefficient calculation with the 30 tested genes resulted in a coefficient of correlation of 0.66 and a coefficient of determination of 0.44 ($P < 0.001$).

Supplementary Table S1. Properties of soils A and K.

	Clay [%]	Silt [%]	Sand [%]	Organic matter [%]	Water content [%]	pH
Soil A	7.4	13.9	78.7	3.7	13.5	3.9
Soil K	3.1	4.3	92.6	4.2	12.6	4.8

Supplementary Table S2. Primer sequences for genes analyzed by RT-qPCR.*

Gene name (MDP ID)	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
elongation factor 1-alpha (MDP0000304140)	*EF1a*	f: GAACGGAGATGCTGGTATGG r: CCAGTTGGCTCCTTCTTCTC	159	103.0	0.998
elongation factor 1-beta 2-like (MDP0000903484)	*EF1b*	f: GAGAGTGGGAAATCCTCTG r: ACCAACAGCAACCAATTTC	138	100.6	0.998
tubulin beta chain (MDP0000951799)	*TUBB*	f: TTCTCTGGGAGGAGGTACTG r: GTCGCATTGTAAGGCTCAAC	147	96.9	0.997
17.1 kDa class II heat shock protein-like (MDP0000700383)	*HSP17.1*	f: CGCCGAGAAGTCATTCAAC r: CACGAACACGTAGGAGTTAG	109	92.8	0.999
17.3 kDa class II heat shock protein-like (MDP0000548065)	*HSP17.3*	f: GACATGCCGGGACTGAAG r: CATTCTCCGGCAACACAAAC	169	94.7	0.998
18.5 kDa class I heat shock protein-like (MDP0000759666)	*HSP18.5*	f: GCTTAAGATCAGCGGAGAG r: CTTCGGAACAGTGACACTTAG	175	98.2	0.997
1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (MDP0000314499)	*ACO1*	f: CGCAGTTGGAGATGAACTTG r: CATGCCGTGATGGACAGTAG	167	101.2	0.996
acidic endochitinase-like (MDP0000218691)	*CHIA*	f: CTGAGTGCTGACATCAGAAC r: GACTGACCACCTAAGAAGTTG	149	99.5	0.999
ankyrin-1-like (MDP0000157404)	*ANK1*	f: CAACAAGAATGGCGAAACTC r: CAAGATCAACCACTTCCATAGG	113	93.2	0.999
biphenyl synthase 2 (MDP0000716308)	*BIS2*	f: CTTGTGGTTTGCGCAGAG r: GTCTGCATGCCACGATTTC	172	96.8	0.997
biphenyl synthase 3 (MDP0000287919)	*BIS3*	f: GGCAAGAAGCAGCATTGAAAG r: CACAACCTGGCATGTCAAC	105	95.3	0.998
biphenyl synthase 4 (MDP0000302905)	*BIS4*	f: CAACAAGCAGCACTGAAAGC r: GATGGGTTAAGGCCAAGGAG	140	102	1
caffeic acid 3-O-methyltransferase-like (MDP0000456277)	*OMTa*	f: CAGGCGTCAGGTGAAAGATG r: GGGTGCTATGGTTGAACATTG	140	95.9	0.999
caffeic acid 3-O-methyltransferase-like (MDP0000745475)	*OMTb*	f: CATCTGAGATCGCAGCTCAC r: GTAGAGCCTCCGGAAACAC	146	99.7	1
cytochrome P450 CYP736A12-like (MDP0000205306)	*B4Ha*	f: GATGTGCACGCTCGAATTAC r: ATTGGCGTCCTCTTGAACAG	106	99.1	0.999
cytochrome P450 CYP736A12-like (MDP0000152900)	*B4Hb*	f: GCTGAGTATGGCCCGTATTG r: AGGAACCCGTCGATTATTGG	156	100	0.999
disease resistance protein At4g27190-like (MDP0000463166)	*DRP*	f: GGAGGATCAAGAGCTTAAAC r: GCCTCTTCTACTGTCTCAAC	123	103.2	0.996
dof zinc finger protein DOF3.5-like (MDP0000308863)	*DOF3.5*	f: CTTTCAGCATCACAACTCATC r: GCAAACACAACAGCCAAATC	105	98.7	1
ethylene-responsive transcription factor 1B-like (MDP0000127134)	*ERF1B*	f: GTCACCTGAATCTTCGTTTG r: GGAAATCAGACCGTAGAGAAG	121	99.7	0.997
ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)	*ERF RAP2.11*	f: TTCCAACAGCCGAAGCAAG r: CTTTGATCTCAGCAACCCATCTC	169	96.4	0.997

Gene name (MDP ID)	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
F-box/kelch-repeat protein At4g12810 (MDP0000708634)	*KFB*	f: GACCTTGCCCACAATCCTC r: GGTGGGTGTAAAGGCTGAAG	145	96.5	1
GATA zinc finger domain-containing protein 10-like (MDP0000922823)	*GATAD10*	f: GCTCGTTTCTGGAGGAGTC r: GATTCCCGCTGTCGTAGAATC	153	99.6	0.998
gibberellin-regulated protein 1-like (MDP0000140078)	*GASA1*	f: CGTTGCAGCTGTGTTCCTC r: CATCTGCATGCCCGAATATGAG	156	101.9	0.997
LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	*MRH1*	f: CGAGGTTTCATGGTTGTTG r: CGCAGAATGACAACAGAATC	163	100.1	0.998
NAC transcription factor 25-like (MDP0000842702)	*NAC25*	f: CGTTGCCACAACCACTAC r: CTCCCATGTCATCTGATATTC	199	100.8	0.995
pectinesterase/pectinesterase inhibitor 41 (MDP0000798705)	*PME41*	f: CAAACCCTACATACTGCAACTC r: GATACTTGTCCACCAAGTTAAGG	126	99.5	0.996
senescence-associated carboxylesterase 101-like (MDP0000837935)	*SAG101*	f: CGTAAACTAGCGATGCAGAAG r: GGACTCCTCCTTGTACCATTC	102	102.1	0.999
thaumatin-like protein 1a (MDP0000552328)	*TL1*	f: ACGGCTTCAACTTGCCTATG r: GCCGCTTTCACTTGAAGTTG	118	92.5	0.992
wound-induced protein 1-like (MDP0000792101)	*WIN1*	f: GATTCCAGCCACACTGACTTC r: CTCCCTGAACTGAGTGATCAAC	150	96	0.999
zinc finger protein ZAT12 (MDP0000595671)	*ZAT12*	f: CAAGAAGCCGAAGCTAATGC r: GTCCGACCGTGAACTCAAG	125	99.2	0.997

*All primers (f: forward, r: reverse) were evaluated in an amplification efficiency test at an annealing temperature of 60 °C (modified after Weiß et al. under revision).

Supplementary Table S3. Gene expression levels analyzed by RT-qPCR in roots cultivated in soils from different nurseries at day 7.*

Class	Gene	Gene name (MDP ID)	Expression γARD ± SD soil A	Expression ARD ± SD soil A	Expression γARD ± SD soil K	Expression ARD ± SD soil K	Ratio soil A	Ratio soil K
Phytoalexin biosynthesis	*BIS2*	biphenyl synthase 2 (MDP0000716308)	0.66 ± 0.6	2.41 ± 0.56	1.18 ± 0.3	21.86 ± 5.78	3.67*	18.5***
Phytoalexin biosynthesis	*BIS3*	biphenyl synthase 3 (MDP0000287919)	2.18 ± 1.25	8.95 ± 1.44	3.82 ± 0.75	40.4 ± 9.95	4.11**	10.58***
Phytoalexin biosynthesis	*BIS4*	biphenyl synthase 4 (MDP0000302905)	0.06 ± 0.05	0.22 ± 0.06	0.08 ± 0.03	1.98 ± 0.7	3.9*	23.95***
Phytoalexin biosynthesis	*OMTa*	caffeic acid 3-O-methyltransferase-like (MDP0000456277)	0.97 ± 0.27	1.24 ± 0.18	2.5 ± 0.45	2.29 ± 0.28	1.27	-1.09
Phytoalexin biosynthesis	*OMTb*	caffeic acid 3-O-methyltransferase-like (MDP0000745475)	1.19 ± 0.4	2.44 ± 0.38	2.89 ± 0.67	8.57 ± 2.12	2.05**	2.96***
Phytoalexin biosynthesis	*B4Ha*	cytochrome P450 CYP736A12-like (MDP0000205306)	0.72 ± 0.36	2.02 ± 0.51	0.73 ± 0.05	4.09 ± 0.63	2.78**	5.62***
Phytoalexin biosynthesis	*B4Hb*	cytochrome P450 CYP736A12-like (MDP0000152900)	0.85 ± 0.4	2.22 ± 0.57	1.04 ± 0.21	6.05 ± 1.63	2.61**	5.83***
Plant defense	*ANK1*	ankyrin-1-like (MDP0000157404)	0.54 ± 0.1	0.5 ± 0.05	0.38 ± 0.06	0.72 ± 0.09	-1.08	1.9***
Plant defense	*CHIA*	acidic endochitinase-like (MDP0000218691)	0.22 ± 0.07	0.63 ± 0.17	0.29 ± 0.13	0.92 ± 0.21	2.8***	3.21***
Plant defense	*DRP*	disease resistance protein At4g27190-like (MDP0000463166)	0.11 ± 0.03	0.1 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	-1.17	1.02
Plant defense	*TL1*	thaumatin-like protein 1a (MDP0000552328)	0.07 ± 0.02	0.3 ± 0.15	0.03 ± 0.03	0.14 ± 0.06	4.32**	4.88*
Regulatory function	*HSP17.1*	17.1 kDa class II heat shock protein-like (MDP0000700383)	0.27 ± 0.07	0.08 ± 0.02	0.02 ± 0	0.02 ± 0	-3.49***	1.11
Regulatory function	*HSP17.3*	17.3 kDa class II heat shock protein-like (MDP0000548065)	0.79 ± 0.23	0.24 ± 0.08	0.04 ± 0.01	0.07 ± 0.02	-3.37***	1.72*
Regulatory function	*HSP18.5*	18.5 kDa class I heat shock protein-like (MDP0000759666)	0.05 ± 0.02	0.01 ± 0.01	0 ± 0	0 ± 0	-3.9***	1.13
Regulatory function	*KFB*	F-box/kelch-repeat protein At4g12810 (MDP0000708634)	0 ± 0	0.02 ± 0.01	0 ± 0	0 ± 0	6.29***	-1.08
Regulatory function	*PME41*	pectinesterase/pectinesterase inhibitor 41 (MDP0000798705)	0 ± 0	0.01 ± 0	0 ± 0	0.01 ± 0	2.39*	3.82***
Regulatory function	*SAG101*	senescence-associated carboxylesterase 101-like (MDP0000837935)	1.69 ± 0.32	1.86 ± 0.14	1.24 ± 0.16	2.17 ± 0.17	1.11	1.75***
Signaling	*ACO1*	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (MDP0000314499)	0.08 ± 0.03	0.03 ± 0.01	0.02 ± 0	0.02 ± 0	-2.35**	-1.44*
Signaling	*DOF3.5*	dof zinc finger protein DOF3.5-like (MDP0000308863)	0.01 ± 0	0.02 ± 0	0.01 ± 0	0.01 ± 0	1.42*	1.22
Signaling	*ERF1B*	ethylene-responsive transcription factor 1B-like (MDP0000127134)	0.01 ± 0	0.02 ± 0	0.01 ± 0	0.11 ± 0.03	3.29**	12.03***
Signaling	*ERF RAP2.11*	ethylene-responsive transcription factor RAP2-11-like (MDP0000177547)	0.04 ± 0.01	0 ± 0	0.06 ± 0.02	0.03 ± 0.01	-12.93***	-2.07**
Signaling	*GASA1*	gibberellin-regulated protein 1-like (MDP0000140078)	0.32 ± 0.05	0.17 ± 0.06	0.03 ± 0.01	0.01 ± 0	-1.95**	-2.66**
Signaling	*GATAD10*	GATA zinc finger domain-containing protein 10-like (MDP0000922823)	0 ± 0	0.02 ± 0	0 ± 0.01	0.01 ± 0	15.06***	2.22*
Signaling	*MRH1*	LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	0.03 ± 0.01	0.02 ± 0	0.01 ± 0	0.01 ± 0	-1.37*	-1.04
Signaling	*NAC25*	NAC transcription factor 25-like (MDP0000842702)	0.01 ± 0	0.02 ± 0	0.07 ± 0.03	0.11 ± 0.03	1.95*	1.65
Signaling	*WIN1*	wound-induced protein 1-like (MDP0000792101)	0.28 ± 0.05	0.11 ± 0.04	0.01 ± 0	0.01 ± 0	-2.44***	-1.42
Signaling	*ZAT12*	zinc finger protein ZAT12 (MDP0000595671)	0.01 ± 0	0.05 ± 0.01	0.02 ± 0.01	0.08 ± 0.02	4.14***	4.77***

*For normalization, *EF1a*, *EF1b* and *TUBB* were used as reference genes. Ratios are represented by the quotient of ARD/γARD with between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Data from Weiß et al. (under revision) was recalculated to obtain comparable expression values. Expression values and resulting ratios (Ratio soil A) are compared to ratios at day 7 from data obtained in this study (Ratio soil K). Differences between treatments were tested using a Welch Two Sample t-test (n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Genes are alphabetically ordered according to their class.

4. Transcriptome profiling in leaves representing aboveground parts of apple replant disease affected *Malus domestica* 'M26' plants

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Abstract

Frequent replanting causes biotic stress due to an altered soil and rhizosphere biome and results in apple replant disease (ARD). The disease is expressed by diminished growth and negatively affects fruit yield and quality. Recent studies aiming at understanding ARD on a molecular level showed that ARD affected plants suffer from oxidative stress. Genes involved in secondary metabolism reactions play an important role in the molecular ARD response of roots. Although the root system has to endure the biotic stress attack in the first place, severe symptoms of ARD can be visualized on aboveground plant parts. The objective of this study was to examine the transcriptomic response of leaves representing the metabolically active aboveground parts of ARD challenged apple plants and to compare results with existing data for roots to generate a more complete picture of ARD affected molecular reactions. For this, biotic stress response genes induced by ARD in roots were studied in RT-qPCR analyses using leaves of ARD sensitive 'M26' plants grown in two ARD soils, also in a time-dependent approach. Furthermore, an RNA sequencing approach employing MACE (massive analysis of cDNA ends) for transcriptome profiling was performed in order to identify further leaf specific candidate genes. RT-qPCR analyses did not reveal major differences in root candidate gene expression, but MACE indicated the upregulation of common biotic stress response genes. However, potential systemic oxidative stress occurred and 'M26' plants did not develop an effective defense response to ARD.

Key words: Apple replant disease; biotic stress response; gene expression; MACE (massive analysis of cDNA ends); *Malus domestica*; oxidative stress; quantitative RT-PCR; RNA-Seq

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Introduction

Worldwide, replanting apple trees (*Malus domestica*) in fruit orchards and tree nurseries leads to the phenomenon known as apple replant disease (ARD) which is expressed by diminished growth already shortly after planting and negatively affects fruit yield and quality (Mazzola and Manici 2012; Liu et al. 2014). Biotic stress in the form of soil-borne fungi or oomycetes of the genera *Cylindrocarpon* (Tewoldemedhin et al. 2011a; Mazzola and Manici 2012; Manici et al. 2013, 2015; Franke-Whittle et al. 2015), *Rhizoctonia* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012; Manici et al. 2015), *Phytophthora* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012) and *Pythium* (Tewoldemedhin et al. 2011b; Mazzola and Manici 2012; Manici et al. 2013) were discussed to be the main driver in ARD while abiotic factors did not significantly influence the disease etiology (Spath et al. 2015). Due to ARD, trees start bearing fruits 2-3 years later than unaffected trees and fail to yield amounts comparable to their healthy counterparts leading to a 50 % reduced profitability throughout the life span of the orchard (Mazzola 1998; Van Schoor et al. 2009). Mazzola (1998) mentioned that over a 10-year period ARD results in a $40,000-per-acre decrease in return of investment in Washington State, USA. While the total value of apple production is valued at $2.5 billion in the USA alone (Brown 2012), both North and South America account for 12.8 % of the worldwide apple production whereas Europe's apple production amounts to 20.7 % (FAOSTAT 2014). Hence, although no specific numbers are available for Europe, ARD is responsible for huge economic losses in Europe as well because examples of ARD incidences have been reported in Austria, Germany, Italy and Poland (Manici et al. 2013; Franke-Whittle et al. 2015; Gastol and Domagala-Swiatkiewicz 2015).

Recent studies have shown that phenolic compounds act as antioxidant substances in response to ARD (Emmett et al. 2014; Henfrey et al. 2015). Although the primary organs being in contact with ARD are roots, oxidative stress even led to a systemic response of ARD affected plants and tolerant genotypes may more efficiently detoxify reactive oxygen species (ROS; Henfrey et al. 2015). Roots of apple seedlings demonstrated an upregulation of genes involved in the secondary metabolism and differentially expressed genes in hormone metabolism after infection by *Pythium ultimum*, one of the important components of ARD (Shin et al. 2014, 2016; Zhu et al. 2014). The importance of secondary metabolism genes in ARD could also be shown for roots of ARD affected apple rootstock 'M26' plants (Weiß et al. 2017). Therein, we analyzed the reaction of apple roots ('M26') to ARD soil on the transcriptomic level. Major differences compared to roots grown in disinfected ARD soil were observed in terms of a downregulation of several genes in the primary metabolism and an upregulation of genes involved in stress response, genes with signaling and regulatory function and genes of the secondary metabolism. In more detail, transcripts of the phytoalexin biosynthesis genes biphenyl synthases, *O*-methyltransferases and biphenyl-4-hydroxylases were detected in significantly higher abundance in roots after 3-7 days of cultivation in ARD soils. Phytoalexin biosynthesis is one way for plants to cope with biotic stress mainly provoked by fungi (Ahuja et al. 2012). However, we hypothesized that the ARD sensitive rootstock 'M26' cannot exploit the phytoalexin defense

mechanism and, rather, results in detrimental conditions for the plant either due to potential toxic levels of phytoalexins or to shaping of a detrimental microbial community if the phytoalexins were excreted (Weiß et al. 2017). It was assumed that tolerant rootstocks may better cope with unfavorable conditions as studies have shown differences in ARD susceptibility between apple rootstocks (St. Laurent et al. 2010; Auvil et al. 2011).

Even though roots are in direct contact with the soil and the potentially harmful microorganisms in ARD, severe visible symptoms of ARD can be detected aboveground. Hence, this study aimed to analyze transcriptomic responses of ARD challenged apple plants in leaves which are the metabolically active representatives of shoots and to compare the results with the existing data for roots. The ARD susceptible genotype 'M26' was employed as a reliable indicator test plant to detect ARD in soils (Yim et al. 2013). RT-qPCR analyses were performed with biotic stress response genes induced by ARD in roots using leaves of plants grown in two ARD soils, also in a time-dependent approach. The fully sequenced genome of apple (Velasco et al. 2010) enabled the application of massive analysis of cDNA ends (MACE) for transcriptome profiling in order to detect additional candidate genes. Hereby, the advantage of MACE compared to more traditional RNA-Seq lies in the generation of exactly one read of each mRNA leading to accurate quantification (Afonso-Grunz et al. 2015; Afonso-Grunz and Müller 2015; Fondevilla et al. 2015; Simm et al. 2015; Zajac et al. 2015; Bojahr et al. 2016). Furthermore, MACE exposes the expression of lower abundant transcripts which cannot be detected by microarrays and RNA-Seq at similar sequence depth (Kahl et al. 2012; Yakovlev et al. 2014; Zawada et al. 2014; Nold-Petry et al. 2015; Zajac et al. 2015).

Materials and methods

Soil origin and disinfection

Soil for the experiments was collected from two tree nurseries in the area of Pinneberg, Schleswig-Holstein, Germany at a depth of 0-25 cm from three field plots each. In March 2014, soil A (53° 42' 18.81'' N, 9° 48' 16.74'' E) was obtained for the transcriptomic profiling (TP) experiment. This soil had the following characteristics: 7.4 % clay, 13.9 % silt, 78.7 % sand, 3.7 % organic matter, 13.5 % water content and pH 3.9. Apple rootstock plants were grown on this site until 2009, succeeded by *Prunus domestica* in 2010, *Cydonia oblonga* in 2011 and the apple rootstock 'M4' in 2012 and 2013 (Yim et al. 2015, 2016). For the time-dependent (TD) experiment, soil K (53° 41' 58.51'' N, 9° 41' 34.12'' E) was acquired in September 2014 with the following characteristics: 3.1 % clay, 4.3 % silt, 92.6 % sand, 4.2 % organic matter, 12.6 % water content and pH 4.8. Cropping history comprised rose rootstock plants from 1980 to 2011, with crop rotation with *Tagetes* starting from 2002 and annual replanting of the apple rootstock 'M4' from 2012 (Yim et al. 2015, 2016). Volumes of 18-20 L of homogenized soil were packed in autoclavable bags (Sarstedt, Nümbrecht, Germany) and half of the respective soil volumes were sent for disinfection via γ-irradiation (BGS – Beta Gamma Service, Wiehl, Germany) at a minimum dose of 10 kGy by which actinomycetes, fungi and invertebrates are

eliminated (McNamara et al. 2003). After disinfection soil bags were stored at 4 °C for 10 days until one day before the start of the experiment.

Plant cultivation and harvest

The highly ARD susceptible apple rootstock 'M26' (Yim et al. 2013, 2015) was propagated and rooted in vitro according to Weiß et al. (2017). For the TP experiment, acclimatization of rooted plantlets lasted four weeks whereas plants for the TD experiment were fully acclimatized after three and a half weeks. Acclimatized plants were potted in 1 L pots containing either γ-irradiated replant soil (γARD) or untreated replant soil (ARD). Each pot was supplied with 2 g L^{-1} of the slow release fertilizer Osmocote Exact Standard 3-4 M (16-9-12+2MgO+TE, http://www.scottsprofessional.com). The TP experiment was started in May 2014 and the TD experiment was launched in December 2014. In May, the conditions in the greenhouse were as follows: 22 °C ± 2.5 °C, 60 % ± 8.7 % relative humidity and a 16 h photoperiod with additional light (if solar radiation fell below 25 klx, provided by SONT Philips Master Agro 400W). Plants grown in December faced the following conditions: 19.3 °C ± 1 °C, 57.7 % ± 6.2 % relative humidity and a 16 h photoperiod with additional light (if solar radiation fell below 25 klx, provided by SONT Philips Master Agro 400W). Irrigation was carried out by hand on a daily basis as required. Furthermore, for plant protection purposes weekly spraying against thrips and spider mites was performed according to horticultural practice. Thirty-five plants per variant were cultivated in the TP experiment of which 25 replicates (= individual plants) per variant were used for subsequent RNA isolation, five plants were used to record dry mass of shoot and root material as well as nutrient analysis of shoot material after 7 days, and the remaining five plants were measured weekly for 56 days to record the shoot length and later on to record dry mass of shoot and root material as well as nutrient analysis of shoot material. In the TD experiment, five different time points for RNA isolation were included, namely 0, 3, 7, 10 and 14 days, and for each time point plant material of 25 replicates (= individual plants) per variant was taken. Together with 10 additional plants the shoot length was measured. In addition, the dry mass of shoot and root material of five plants was recorded at each time point as well as at the end of the experiment, plus 35 plants were measured weekly over the duration of the experiment for a total of 56 days.

For RNA isolation plant material was harvested after 7 days of cultivation in either γARD or ARD soil for the TP experiment whereas in the TD experiment harvest was performed at days 0 (acclimatized plants before potting), 3, 7, 10 and 14. The three youngest fully developed leaves were collected from each plant, put into 2 ml tubes (Sarstedt, Nümbrecht, Germany) and frozen in liquid nitrogen. Until homogenization of plant material, samples were stored at -80 °C.

Nutrient analysis

Nutrient analysis in shoot material of the TP experiment was performed after drying samples in a dry oven at 70 °C for 3 days. Samples were homogenized using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) for 1 min at 30 Hz. For the analysis five biological replicates were used and 50 mg

per sample were weighed into small beakers. The samples were then dry-ashed over night at 480 °C. After the samples were cooled down, 1 ml of 6 M HCl with 1.5 % (w/v) $ClNH_4O$ was added to the samples for dissolving. After shaking and incubation at room temperature for 5 min, the solution was diluted with 9 ml ddH_2O. The dissolved samples were gently shaken and filtered through filter paper with a pore diameter of 2 µm. Analysis was done employing an ICP-MS method (Führs et al. 2010). To measure C, N and S in the samples, 10 to 15 mg of dried plant material was weighed into small tin vessels and an equal amount of wolfram was added to the samples before they were analyzed with a CNS method (Kowalenko 2001).

RNA extraction and first strand cDNA synthesis

Leaves of five plants were combined in a biological pool (= biological replicate). In sum, five pools were analyzed for each soil, variant and time point. Homogenization and cell disruption in liquid nitrogen cooled steel cups was performed using a mixer mill (Mixer Mill MM400, Retsch, Haan, Germany) with liquid nitrogen cooled steel beads (ø 1.5 cm) for 1 min at 30 Hz.

Using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) with an extraction buffer for phenol containing plants according to the manufacturer's instructions, RNA was extracted from 50 mg homogenized leaf material weighed into 2 ml tubes (Sarstedt, Nümbrecht, Germany). RNA yield and quality were checked by a spectrophotometer (Nanodrop 2000c, Peqlab, Erlangen, Germany). Genomic DNA was removed via in solution DNA digestion using 1 µg of total RNA and DNase I (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions and the resulting RNA was assessed for integrity on a 1 % agarose gel. Aliquoted RNA was stored at -80 °C until further proceeding.

First strand cDNA synthesis to reversely transcribe total RNA was performed by employing the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions with random hexamer primers. Aliquoted cDNA samples for RT-qPCR analyses were stored at -80 °C.

Primer design and quality check

Primer design of *TL1b*, *KFB1*, *EIL2*, *EIL3*, *IAA8* and *PTI5* was comprised of the following parameters: A primer length of 18-24 bp, a resulting fragment length of 100-200 bp, T_M of 57-63 °C, GC content of 40-60 %, no more than triple repeats (e.g. GGG), GC clamp at the 3' end but no more than two times GC in the last five base pairs, no dimers, no self-complementarity and no hairpin structures. Clone Manager 9 Demo (Sci-Ed Software, Denver, CO, USA) was used to check primers according to set parameters. Complementary sequences to get reverse primers were obtained from GeneRunner (Gene Runner, http://www.generunner.net/). Specificity of primers was confirmed in the *Malus x domestica*.v1.0-primary.mRNA database (https://www.rosaceae.org). Primer sequences of the other tested genes were derived from Weiß et al. (2017). All primers were subjected to a quality check via RT-qPCR efficiency tests to monitor sufficient amplification efficiency and specificity using a

real-time PCR cycler (CFX Connect™, Bio-Rad, Hercules, CA, USA). Mixed cDNA samples of equal amounts of γARD and ARD cDNA were analyzed in a dilution series of 1:10, 1:50, 1:100, 1:500 and 1:1000. The dilution samples were tested with two technical replicates using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 200 nM for each forward/reverse primer at 60 °C annealing temperature. The protocol for the efficiency tests was as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The program was ended after a melt curve analysis from 65 °C to 95 °C with an increment of 0.5 °C for 5 s at each step. Data was recorded with the Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA) and amplification efficiencies were calculated. Primer pairs with amplification efficiencies of 90-110 % showing specific amplification according to the melt curve analysis are listed in Table 1.

RT-qPCR gene expression analyses

Candidate genes and reference genes (*EF1a, EF1b* and *TUBB* according to expression stability) were analyzed with a real-time PCR cycler (CFX Connect™, Bio-Rad, Hercules, CA, USA). The 1:10 cDNA dilution was mixed with primer combinations (200 nM for each forward/reverse primer) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Data was recorded with the Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA). The protocol for RT-qPCRs was as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The program was ended after a melt curve analysis from 65 °C to 95 °C with an increment of 0.5 °C for 5 s at each step. Five biological replicates and two technical replicates per soil, variant and time point were analyzed for gene expression. Normalized expression was calculated according to Pfaffl (2001).

Transcriptomic analysis via MACE

MACE (Kahl et al. 2012) was employed for transcriptomic analysis of leaf material obtained in the TP experiment. Three biological replicates per variant were analyzed after sending extracted RNA to GenXPro GmbH (Frankfurt am Main, Germany). Data was examined using CLC Genomics Workbench 8.5.1 (Qiagen, Hilden, Germany). Sequences have been submitted to the NCBI Sequence Read Archive (SRA) under the accession number SRP097602. Removal of sequenced bases resulting from cDNA synthesis primers was achieved by using a poly-A adapter (Nold-Petry et al. 2015). The *Malus x domestica*.v1.0-primary.mRNA database obtained from https://www.rosaceae.org (11.11.2014) was downloaded to map trimmed sequences. Parameters for mapping were as follows: Mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, strand specific = both, maximum number of hits for a read = 10 and expression values = total counts. Mapped genes present in all three biological replicates of either variant resulted in a data set (referred to as L3P) which was later used for comparison with root data (genes that were present in both of the analyzed biological replicates referred to as R2P; modified Weiß et al. 2017) to find shared genes. For the filtered data set (referred to as FTP), CLC's implemented Baggerley's test was used to compare mapped sequences with a false discovery rate (FDR) adjusted $P < 0.05$ according to the ratio of ARD

to γARD of trimmed read numbers (ARD γARD^{-1}). Resulting ratios were $\log_2$ transformed and mapped sequences were ordered according to their ratio in Excel 2010. In addition, for visualization purposes ratios were also calculated using γARD as the denominator and values between 0 and 1 were transformed by multiplying with the negative reciprocal value to attain similar results to a $\log_2$ transformation. Negative values indicated a downregulation of transcripts in ARD samples compared to γARD samples, whereas positive values specified an upregulation of transcripts in ARD samples. MapMan (Thimm et al. 2004) was used to assign functional categories to transcripts. Corresponding sequences were blasted using default parameters for BLASTN of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to obtain potential gene functions. MACE data was validated via RT-qPCR (see *RT-qPCR gene expression analyses*) with biotic stress response genes induced by ARD in roots (Weiß et al. 2017) in addition to *TL1b*, *KFB1*, *EIL2*, *EIL3*, *IAA8* and *PTI5*.

Statistical analyses

The statistics program R 3.3.2 (R Development Core Team 2008) was used for statistical analyses with $\log_2$ transformed data. At a $P < 0.05$ means of measured or calculated parameters between soil variants (γARD, ARD) were compared using the Welch Two Sample t-Test (Welch 1947). Using a Tukey multiple comparisons test adjusted for heterogeneous variances if needed (Tukey 1949; Herberich et al. 2010) at a $P < 0.05$, different time points within one soil variant were compared. For soil A (TP experiment), variables were scaled to unit variance and components were built according to correlations among variables in a principal component analysis (PCA, Lê et al. 2008). Multivariate differences between variables were tested using analysis of similarities (ANOSIM) employing euclidean distance in a permutation test with 9999 permutations (Clarke 1993). Contribution to the separation of groups by variables was expected at a value > 0.69 % according to R 3.3.2. Genes exceeding this threshold were analyzed by Past 3.14 (Hammer et al. 2001) in a Pearson product-moment correlation matrix (Pearson 1895) together with the shoot length of samples. Correlation between gene expression data obtained at day 7 of the TP experiment (soil A) and the TD experiment (soil K) was tested using the Pearson product-moment correlation coefficient (Pearson 1895) which was calculated in Microsoft Excel 2010.

Results

ARD had a negative effect on 'M26' shoot and root dry mass

Disinfection of ARD soil led to higher shoot and root dry mass of 'M26' plants at the end of the experiment (Fig. 1). At day 56, plants cultivated in soil A had a shoot dry mass of 3.33 ± 0.4 g in disinfected soil variants which was almost double the mass (= + 85 %) found in ARD variants with 1.8 ± 0.35 g. The root dry mass was also significantly affected as roots of γARD variants weighed 0.73 ± 0.17 g compared to ARD variants with 0.45 ± 0.11 g. Even stronger effects were observed for plants grown in soil K as shoot dry mass for γARD variants was 3.53 ± 0.39 g (= + 123 %) compared

to 1.58 ± 0.23 g for untreated variants. Also the root dry mass differed in soil K with 1.03 ± 0.15 g recorded for disinfected variants and 0.63 ± 0.14 g for ARD variants. While the dry mass did not differ at day 7 for both soils the shoot length was already affected at this date in soil K, whereas soil A revealed significant differences between the variants in their shoot length at day 14 (Supplementary Table S1).

ARD hardly affected nutrient contents in 'M26' plants

Due to the diminished growth of plants cultured in ARD soil A, also a nutrient analysis of shoot material was performed. The analysis did not demonstrate pronounced differences between plants grown in either γARD or ARD soil (Table 2). At day 7, only Mg, Na and S were found in slightly higher concentrations of ARD samples: Disinfected soil resulted in 1.68 ± 0.18 mg/g, 0.81 ± 0.17 mg/g and 3.23 ± 0.3 mg/g dry mass, respectively, whereas ARD samples exhibited 1.96 ± 0.14 mg/g, 1.18 ± 0.18 mg/g and 3.86 ± 0.37 mg/g dry mass, respectively. After 56 days of cultivation ARD samples demonstrated marginally lower B (0.02 versus 0.03 mg/g dry mass), C (25.38 versus 27.48 mg/g dry mass) and P (0.83 versus 0.97 mg/g dry mass) concentrations while Ca (7.37 versus 5.43 mg/g dry mass) and Sr (0.04 versus 0.02 mg/g dry mass) were present in slightly higher concentrations.

Expression analyses of selected genes by RT-qPCR

The selection of genes included in the RT-qPCR analyses in leaves included first biotic stress response genes that had been shown to be induced by ARD in roots grown in soil A (Weiß et al. 2017). Secondly, biotic stress response genes found to be differentially expressed in L3P transcriptomic data of leaves of plants grown on soil A were added (*TL1b*, *KFB1*, *EIL2*, *EIL3*, *IAA8* and *PTI5*). A time-course experiment was performed in soil K to obtain more detailed information on the expression of the chosen genes over time but also to test for potential conservation of gene expression among ARD soils with different properties and cropping history at day 7. For RT-qPCR analyses, elongation factor 1-alpha (*EF1a),* elongation factor 1-beta 2-like (*EF1b)* and tubulin beta chain (*TUBB*) were used as reference genes. Overall, the tested genes demonstrated low expression levels in soil K (Table 3). Moreover, genes which were strongly affected over time in roots cultivated in soil K showed less pronounced differences in expression behavior in leaves (Supplementary Fig. S1-2). However, most phytoalexin biosynthesis genes, although being expressed at a much lower level than in roots, started to increase in expression at day 14 (Supplementary Fig. S1). Calculating the Pearson product-moment correlation coefficient showed a significant positive correlation between gene expression in leaves of plants cultivated in soil A and soil K at day 7 with $R = 0.65$ and $P < 0.01$, but none of the selected genes were significantly regulated at this time point in soil K (Table 3).

Transcriptomic profiling of leaves in ARD

Transcriptome profiling was performed via MACE to examine molecular reactions in leaves in response to ARD in soil A at day 7. Samples were analyzed and resulted in 9 to 14 million reads with an average length of 109 to 110 bp (Table 4). Approximately 30 % of all reads could be specifically mapped (unique mapped reads) to the draft genome of apple (Velasco et al. 2010) while 2 % of all reads matched to more than one sequence. These non-specifically mapped reads were excluded from further analyses.

In a previous study (Weiß et al. 2017) ARD affected roots cultivated in soil A were analyzed via MACE. The comparison of differentially expressed genes identified in both tissues (L3P vs. R2P, see 2.7.) resulted in 40 genes (2.6 %) in γARD samples (of which 30 genes could be matched to NCBI entries, Supplementary Table S2) and 36 genes (2.7 %) in ARD samples (of which 27 genes could be matched to NCBI entries, Supplementary Table S3) that were higher expressed in both, roots and leaves (Fig. 2). Interestingly, while in ARD samples almost the same amount of genes was higher expressed in leaves and roots (46.6 % and 50.7 %, respectively; Fig. 2B), in root γARD samples 82.2 % of the identified genes were higher expressed compared to leaves with 15.3 % (Fig. 2A) but none of the shared genes were part of the relevantly contributing genes in the PCA of leaves (Table 5) and only few were part of the FTP data set (Supplementary Table S4).

To verify the leaf MACE data, the RT-qPCR data set of the selected genes of interest (Table 1; Table 3) was used. There was a strong correlation between the MACE and RT-qPCR datasets as indicated by the Pearson product-moment correlation coefficient at $R = 0.83$ and $P < 0.001$ pointing to the reliability of the MACE data.

FDR corrected filtering for differential expression at $P < 0.05$ resulted in 726 genes for further consideration of which 642 genes could be matched to NCBI entries (FTP data set, Supplementary Table S4). Compiling genes with at least 2-fold regulation or which were found exclusively in γARD or ARD samples and which revealed a relevant contribution, spanning all dimensions, to the separation of γARD and ARD samples in the PCA (ANOSIM: $R = 1$) resulted in a set of 30 genes which were associated to MapMan functional categories (Table 5). Captivatingly, only 6 genes showed relevant contributions and uniqueness in γARD samples or demonstrated high regulations which included DNA topoisomerase 2-like (ratio: -11.76), heat shock protein 90-2-like (-3.59), protein RETICULATA-RELATED 1 (-2.16), phospholipase A1-IIdelta-like (only γARD), anthocyanidin 3-O-glucosyltransferase 5-like (only γARD) and heat shock factor protein HSF30-like (only γARD).

The remaining relevantly contributing genes were found in ARD samples. Here, UDP-glucose flavonoid 3-O-glucosyltransferase 7-like (14.93) and major allergen Mal d 1-like (14.89) revealed very high regulations whereas cyclin-D5-1-like and F-box protein SKIP2-like were only induced in ARD samples. Protein processing genes involved elongation factor 1-gamma-like (2.01), AAA-ATPase ASD, mitochondrial-like (2.44), mechanosensitive ion channel protein 6 (3.44) and ATP-dependent zinc metalloprotease FTSH 9 (3.92). Transcription factor HEC2-like (2.08), two-component response

regulator ORR9-like (2.43), ethylene-responsive transcription factor ERF113 (2.60), major allergen Mal d 1-like (3.01) and probable WRKY transcription factor 9 (5.71) mapped to RNA processing genes. Furthermore, the signaling genes probable high-affinity nitrate transporter 3.2 (3.74), calcium-binding protein CML27 (2.11) and cysteine-rich receptor-like protein kinase 10 (2.60) as well as the stress associated gene putative disease resistance protein RGA3 (2.37) were relevantly contributing to the separation of samples. Additional genes included GDSL esterase/lipase 5-like (2.08), caffeic acid 3-*O*-methyltransferase-like (2.76), translation initiation factor IF-3-like (2.20), mechanosensitive ion channel protein 1 (2.23), ABA-inducible protein PHV A1-like (2.52), putative UPF0481 protein At3g02645 (2.60) and transcription factor PRE6-like (5.69). Half of the contributing genes also showed a significant correlation to the shoot length (Table 5, Supplementary Fig. S3).

Discussion

Due to the phase-out of fumigants controlling ARD, it has increasingly become more important to better understand the complex mechanisms in plants suffering from this worldwide phenomenon. Our recent studies uncovered molecular responses of apple roots dealing with ARD (Weiß et al. 2017). Roots are in direct contact with the soil and the microorganisms leading to ARD, but the first observable symptoms can be visualized aboveground as plants faced with ARD show diminished growth compared to plants cultivated in disinfected ARD soil (Yim et al. 2013, 2015, 2016; Henfrey et al. 2015; Weiß et al. 2017). Hence, besides probable limitations in water and nutrient uptake due to root damage, early molecular and physiological changes in affected roots likely trigger signal cascades leading to this detrimental early growth reduction. This study aimed to examine the transcriptomic response in leaves of ARD affected apple ‘M26’ plants on ARD soils of different origins, characteristics and cropping histories to generate a more detailed picture of molecular reactions in the plant when faced with ARD. The ARD susceptible apple rootstock ‘M26’ was used due to its proven strong reaction towards ARD (Yim et al. 2013, 2015; Weiß et al. 2017) as well as the need to investigate molecular ARD reactions in a challenged genotype.

ARD induced growth reduction of apple rootstock ‘M26’

As shown in previous studies (Yim et al. 2013, 2015; Weiß et al. 2017), ARD affected apple rootstock ‘M26’ plants were shorter in shoot length (Supplementary Table S1) and, furthermore, revealed lower biomass after 56 days of cultivation (Fig. 1). While plants grown on soil A showed significant shoot length differences between variants after 14 days of cultivation, ‘M26’ plants cultivated in soil K were already significantly affected after 7 days (Supplementary Table S1). This observation leads to the conclusion that soil K was more severely affected by ARD. In addition, this point gets emphasized by the observation that plants in soil K were taller in the beginning but smaller at the end of the experiment compared to plants cultivated in soil A. However, it has to be noted that experiments employing soil A were conducted in summer 2014 whereas soil K was used in winter 2014. Even though plants were cultivated under greenhouse conditions, mean temperatures differed about 3 °K

between seasons. Nevertheless, γ-irradiation of soils was obviously able to effectively eliminate unfavorable microorganisms from ARD soils as shown by majorly improved overall growth. Microbial community shifts in disinfected soil can potentially improve growth via increasing genera with conceivably beneficial features (Yim et al. 2015). Moreover, abiotic factors in the form of changes in nutrient contents leading to the observed growth differences may be excluded as shoot material grown in soil A did not show differences regarding nutrient contents between variants (Table 2, ANOSIM: $R = 0.048$) which is in line with results reported earlier (Spath et al. 2015).

Comparison of ARD challenged 'M26' leaves in soil A and soil K

Genes that had been selected due to their regulation in ARD affected roots or their involvement in biotic stress response were found to be expressed on a much lower level and only with minor differences between ARD and disinfected ARD soil compared to roots (Table 3). In soil A, only *HSP17.1*, *HSP17.3* and *HSP18.5* were significantly regulated. The same HSP genes were also significantly downregulated in ARD affected roots of apple rootstock 'M26' plants (Weiß et al. 2017). On the one hand, this could potentially highlight their importance in maintaining metabolism and growth as they were positively correlated with the shoot length (Weiß et al. 2017) and it emphasizes the significance of these genes in protein stabilization and refolding of proteins under stressful conditions (Hüttner and Strasser 2012; Park and Seo 2015) as well as their contribution to innate immunity (Liu and Howell 2010; Park and Seo 2015). However, on the other hand, it has to be mentioned that plants for the TP experiment were collected in summer, and one day before harvest as well as on the collection day temperatures were 3 to 5 K higher compared to the days before. This could actually mean that the expression of HSP genes were indeed a reaction to heat stress, which seemed to be impeded then under ARD conditions.

The candidate genes used here were involved in phytoalexin biosynthesis, plant defense, regulatory function and signaling which are often related to biotic stress response and which showed strong reactions in roots of ARD plants (Weiß et al. 2017). We were interested if these genes also were expressed in a systemic response in leaves as ARD challenged plants react with a systemic response – roots are in direct contact with the biotic stress but aboveground tissue is negatively affected as well – however, this has to be negated for most cases based on the findings of our study. One indication for a delayed systemic response might be deduced from the increase of many phytoalexin genes starting at days 10-14 (Supplementary Fig. S1). Overall, the results point to the analyzed genes playing a major role in ARD reaction of roots but not of leaves.

Gene expression in leaves was also compared for plants grown in soil A with those in soil K in order to identify genes of conserved expression patterns among soils with differing origins, soil characteristics and cropping histories. Results of gene expression analyses in root materials suggested mostly a conservation of expression of these candidate genes between the same two soils, but few genes were found to be soil-specifically expressed (Weiß et al. in preparation). At day 7, only a low

positive correlation between gene expression in leaves of plants cultivated in soil A and soil K ($R = 0.65$ and $P < 0.01$) could be determined but it has to be noted that none of the selected genes was significantly regulated at this time point in soil K (see above).

Transcriptomic response of ARD challenged 'M26' leaves in soil A

To identify early affected molecular reactions involved in ARD, the harvest time point for transcriptomic profiling in soil A was set to 7 days after planting. In addition, stress reactions due to repotting of plants after acclimatization should not impede reactions to soil treatments. Furthermore, transcriptomic changes as a response to ARD could be detected already after 7 days in roots of 'M26' plants (Weiß et al. 2017) and therefore leaves were analyzed in the early stages of ARD as well. MACE was employed as the method of choice, because it allows for exact quantification of transcripts and detection of low abundant transcripts (see introduction). For example in human samples more than 200 million reads are prerequisite to identify a complete transcriptome with all potential isoforms using RNA-Seq while MACE discovers all conceivable isoforms with less than 10 million reads and therefore presents a resourceful deep-sequencing method for transcriptome profiling (Zajac et al. 2015). In this study, MACE generated parameters within ranges also reported for other studies: The number of reads with 9 to 14 million (Table 4) was higher in comparison to tomato pollen with 3 to 6 million reads (Bokszczanin et al. 2015) and similar to tomato leaves with 12 to 15 million reads (Fragkostefanakis et al. 2015) while in apple roots the number of reads ranged from 12 to 22 million (Weiß et al. 2017). Hence, an appropriate coverage was achieved. Furthermore, the aforementioned studies showed percentages of mapped reads for apple roots with 32 % and tomato pollen with 30 to 45 % which is similar to the reported 30 % in this study and higher compared to tomato leaves with 10 to 11 %. The average length of trimmed reads between 109 and 110 bp was higher compared to previous reports stating a range of 50 to 100 bp (Kahl et al. 2012; Müller et al. 2014; Zajac et al. 2015).

RT-qPCR gene expression data validated MACE data as tested candidate genes were regulated with the same tendency as indicated by the Pearson product-moment correlation coefficient ($R = 0.83$, $P < 0.001$). A relatively small proportion of 2.6-2.7 % of all differentially expressed genes found in roots of ARD affected apple rootstock 'M26' plants (Weiß et al. 2017) was also found in leaf material (Fig. 2). Especially for genes with higher expression in γARD samples in leaf and root material, it was striking that 16 out of 30 genes were HSP genes or transcription factors. Their downregulation in ARD samples then would point to an impaired abiotic stress response as plants were already occupied in dealing with biotic stress due to ARD. To exclude this possible abiotic stress factor, future experiments should be performed in winter or in climate chambers where controlled conditions can be better established. In this regard, also the presence of two multiprotein-bridging factor 1c-like genes which were higher expressed in γARD samples of leaf and root material point to

this fact as it could be shown that transgenic plants expressing this gene were more tolerant to environmental stress including biotic stress (Suzuki et al. 2005).

Impaired stress response and potential oxidative stress in ARD affected plants

In total 726 genes passed the filtering for false positives (FDR at $P < 0.05$, FTP data set, Supplementary Table S4) and 30 genes of these showed a relevant PCA contribution and were used for MapMan classification. Here, ARD samples involved genes in protein processing, RNA processing and signaling (Table 5). These classes represent the oft-enunciated responses to biotic stress (Broekaert et al. 2006; Sato et al. 2010; Tsuda and Katagiri 2010; Ahuja et al. 2012; Shin et al. 2014; Zhu et al. 2014). In this regard, the 2.01 upregulation of elongation factor 1-gamma-like responsible for protein synthesis is a first hint at stressful conditions for ARD plants but also the 2.08 times upregulation of GDSL esterase/lipase 5-like which, due to its multifunctionality, also acts in biotic stress response (Chepyshko et al. 2012). Also the stress associated gene putative disease resistance protein RGA3 which was 2.37 times upregulated further hints at stressful conditions in ARD soils. It has been reported that plants try to alleviate detrimental conditions by increased protein synthesis (Kosová et al 2011) and the 2.20-fold upregulation of the translation initiation factor IF-3-like as well as the induced F-box protein SKIP2-like gene which is involved in protein ubiquitination as part of protein modification under biotic stress support this fact (Ul Hassan et al. 2015). Even the 3.92-fold upregulation in ARD samples of ATP-dependent zinc metalloprotease FTSH 9 could hint at stress responses in ARD soil as proteins could be degraded at a higher rate and it was discussed that protein degradation is upregulated under stress due to protein modification as a reaction to oxidative stress (Berlett and Stadtman 1997; Bartels and Sunkar 2005). AAA-ATPase ASD, mitochondrial-like which has also been reported to act as a metalloprotease (Baek et al. 2011) was upregulated 2.44 times as well but this gene also shows a response to abscisic acid (ABA) and acts in hypersensitive responses (Baek et al. 2011) which would be in line as a typical response to biotic stress (Zhu et al. 2014). Moreover, an ABA-inducible protein PHV A1-like was 2.52 times upregulated in ARD samples and potentially hints at ABA being involved in the insufficient defense response in ‘M26’ plants. ABA is reported commonly to play a role in biotic (as well as abiotic) stress responses (Zhu et al. 2014). This already hints at the large amount of energy that ARD challenged ‘M26’ plants have to invest into defense mechanisms, hence, less energy is available for plant growth resulting in the diminished habitus of these plants compared to γARD variants. This so called immunity growth tradeoff implied by either induced or constitutive resistance, which begs the question if the cost-benefit for growth under induced resistance is better for the plant than constitutive resistance (Heil and Baldwin 2002), obviously has no advantage for the induced non-working defense of the susceptible genotype ‘M26’.

An additional sign of biotic stress could be due to the upregulation of two mechanosensitive ion channel protein genes. Increase of calcium ions is one of the early signaling mechanisms in plants to respond to biotic stress (Kurusu et al. 2010). For transport through membranes, ion channels are

responsible and in our study we observed two genes being upregulated 2.23 and 3.44, respectively, namely the mechanosensitive ion channel protein 1 and 6. It has been shown that cytosolic calcium ions increased after elicitation with fungal elicitor Pep-25 in *Arabidopsis thaliana* which led to an oxidative burst (Hu et al. 2009), and in our study we found the 2.11 upregulation of probable calcium-binding protein CML27. Another sign of affected membranes in ARD samples is the 2.60 upregulation of putative UPF0481 protein At3g02645 acting as transmembrane protein with unknown function according to UniProt.

Another typical response to stress is nitrate reallocation from leaves to roots (Chen et al. 2012) which in this study may be hinted at as well by the 3.74 times higher expression of high-affinity nitrate transporter 3.2. Low nitrate concentrations can induce nitric oxide formation (Modolo et al. 2005) which is a common signaling molecule in biotic stress response (Zhu et al. 2014). In addition, it could be shown that the nitrate high affinity transport system is used for systemic signaling for environmental sensing and signal transduction from roots to shoots to combine plant growth and nutrient availability (Little et al. 2005; Krouk et al. 2010; Gojon et al. 2011).

WRKY transcription factors often are upregulated under biotic stress and play an important role in the plant innate immune system (Rushton et al. 2010; Chi et al. 2013). Here, probable WRKY transcription factor 9 was 5.71 times higher expressed in ARD samples. Higher transcript abundance of WRKY transcription factors was also found in *Erwinia amylovora* infected apple plants (Kamber et al. 2016). Just as kinases can be involved in signal transduction pathways triggered by biotic stress (Afzal et al. 2008; Shin et al. 2016) – here, cysteine-rich receptor-like protein kinase 10 was upregulated 2.60 times – WRKY transcription factors are also part of signal transduction pathways via regulation of other WRKY transcription factors but also hormones that play a vital role in biotic stress response such as ethylene – here, the ethylene-responsive transcription factor ERF113 was 2.60 times upregulated – jasmonic and salicylic acid which in turn coincidences with the generation of ROS (Bakshi and Oelmüller 2014). It could be shown that ARD affected apple plants suffer from oxidative stress resulting in a systemic response towards ARD (Henfrey et al. 2015), which may be an explanation of probable WRKY transcription factor 9 being higher expressed in leaves of this study.

The involvement of genes in ROS signaling was also highlighted by two major allergen Mal d 1-like genes which showed 3.01 and 14.89 times higher expression in ARD samples, respectively, and belong to the class of genes responsible for the expression of pathogenesis-related (PR) proteins, in particular PR-10 (Fernandes et al. 2013). PR-10 proteins are formed upon biotic stress response amongst others and help in general defense mechanisms serving a protective role as they are invoked and accumulate around infection sites by bacteria and fungi plus they are induced by oxidative stress as well (Fernandes et al. 2013).

Transcriptomic results of ARD challenged roots suggested that phytoalexin biosynthesis may potentially increase the overall oxidative stress provoked by ARD microorganisms as a failed defense response which in turn may have led to other genes involved in scavenging ROS being higher

expressed under ARD conditions as well (Weiß et al. 2017). In this context, caffeic acid 3-*O*-methyltransferase-like was upregulated 2.76 times in ARD leaf samples. It is potentially involved in phytoalexin biosynthesis as an isoform leading to biphenyl and dibenzofuran production (Khalil et al. 2015). Since other phytoalexin biosynthesis genes were observed to be upregulated starting from day 14 (Supplementary Fig. S1), analyses of phytoalexin contents in leaves of plants subjected to ARD should be conducted starting at this time point.

Studies examining the transcriptomic response of apple seedling to the infection with *P. ultimum*, which is one of the many potential causal agents of ARD, revealed genes functioning in ROS and antioxidant metabolism as well as kinase signaling (Shin et al. 2016). It was suggested that ascorbate and glutathione as part of ROS scavenging systems may potentially be involved in ARD as well. In our previous studies working with the same soil and roots, it could be shown that genes associated to the ascorbate and glutathione redox systems were upregulated in ARD samples leading to the assumption that a non-sufficient ROS scavenging system in the ARD susceptible rootstock 'M26' might be participating in the strong negative response of plants in ARD soil (Weiß et al. 2017). Henfrey et al. (2015) were able to show that ARD affected apple seedlings demonstrated a systemic response regarding ROS scavenging as application of additional stress in leaves did not further increase the level of oxidative stress which may serve as an explanation why leaves analyzed in this study demonstrated upregulation of two major allergen Mal d 1-like genes in leaves when only roots were in direct contact with biotic stressors. Furthermore, the 14.93 times upregulation of UDP-glucose flavonoid 3-O-glucosyltransferase 7-like in ARD samples emphasizes this point. This gene is involved in anthocyanin production and anthocyanins have been reported to act in defense against pathogens as antimicrobial metabolites and exhibiting antioxidant activity (Vanderauwera et al. 2005; Shih et al. 2007; Hu et al. 2011). This further gets supported by the fact that RNA processing seemed to be affected as well, as shown by the 2.08 and 5.69 upregulation of transcription factor HEC2-like and transcription factor PRE6-like, respectively, as part of the bHLH (basic/helix-loop-helix) transcription superfamily and its involvement in regulation of transcription (Toledo-Ortiz et al. 2003). One prominent member of this family is the *R* gene product Lc which participates in anthocyanin synthesis in maize (Ludwig et al. 1989). However, anthocyanidin 3-O-glucosyltransferase 5-like was only induced in γARD samples which either shows the inconsistent response of ARD challenged 'M26' plants or points to possible misannotations of genes in the apple genome.

Interestingly, regulation of transcription could also be found for the 2.43 upregulated two-component response regulator ORR9-like which is involved in the cytokinin-activated signaling pathway according to UniProt. It was already discussed that cytokinin signaling may play an exceptional role in the defense response in apple roots defending against *P. ultimum* which is also involved in ARD (Shin et al. 2016). In regard of root to shoot signaling this represents an interesting aspect of ARD reactions as cytokinin reportedly interacts with other signal transduction pathways in

regulatory networks including biotic stress responses involving salicylic acid signaling (Argueso et al. 2009; Choi et al. 2010, 2011; Hwang et al. 2012).

The diminished shoot length of ARD samples may already explain that protein RETICULATA-RELATED 1 involved in differential development of bundle sheath and mesophyll cell chloroplasts (Kinsman and Pyke 1998) was downregulated 2.16 times in ARD samples. Additionally, a phospholipase A1-IIdelta-like gene was found to be expressed only in γARD samples. Phospholipases participate in the phospholipid metabolism and are vital signaling molecules (Xu et al. 2016). Furthermore, phospholipases play a major role in jasmonic acid production through release of linolenic acid from membrane lipids (Narváez-Vásquez et al. 1999; Ishiguro et al. 2001).

In addition, the transcriptional regulatory system of ARD plants may have been negatively affected by ARD as DNA topoisomerase 2-like was 11.76 times downregulated and is involved in DNA replication and transcription (Lodish et al. 2000). It has been reported that stress signals result in blocking important metabolic processes including DNA replication, transcription, mRNA export and translation until the cells recover (Biamonti and Caceres 2009). Moreover, topoisomerase activity controls supercoil structures at the 3′ end of genes and thereby contributes to nucleosome disassembly and efficient transcription termination while decreased activity leads to a less accessible chromatin structure, hence, obstructing signals for transcript termination (Durand-Dubief et al. 2011). In this regard it is interesting that cell division was affected as shown by the induction of cyclin-D5-1-like in ARD samples. The function of the cell division cycle is to allocate copies of the genome to daughter cells and genome instability would occur if cells initiate mitosis when chromosomes are only partially replicated leading to cell death (Rhind and Russell 2012). Nevertheless, it was also reported that one of the plant's defense strategies is priming which is defined as the activation of faster and stronger defense signals through potentiating the basal immune system which might be facilitated by chromatin remodeling (Conrath 2011; Camañes et al. 2012). The downregulation of DNA topoisomerase 2 in ARD samples, therefore, might indicate a suppression of this defense mechanism in 'M26' plants faced with ARD.

In ARD samples the heat shock protein 90-2-like gene was downregulated 3.59 times and the heat shock factor protein HSF30-like was only induced in γARD samples. As mentioned earlier, this could emphasize the importance of HSP genes in maintaining metabolism and growth as well as protein stabilization and refolding of proteins under stressful conditions (Hüttner and Strasser 2012; Park and Seo 2015) plus their contribution to innate immunity (Liu and Howell 2010; Park and Seo 2015) but due to higher temperatures before and at harvest the expression of HSP genes could also mean a reaction to heat stress.

Conclusion

This study aimed at a better understanding of ARD on a molecular level in the whole plant to get a more complete picture of the disease etiology. Although plants react with a systemic response to ARD

as indicated by diminished shoot growth while the roots are in direct contact with the biotic stressors, analyzed genes did not show a systemic response in below- and aboveground tissue of the same age. However, phytoalexin biosynthesis genes hint at a delayed systemic response on the transcriptomic level. Nevertheless, indications have been found that ARD challenged 'M26' plants react to the biotic stress in the form of potential systemic oxidative stress. Many genes involved in biotic stress reactions have been found but the obvious lack of their effect leads to the assumption that ARD challenged 'M26' plants cannot fully take advantage of the defense mechanisms leading to the observed growth depressions in ARD variants as discussed in the aspect of immunity growth tradeoff. Future studies should investigate the expression of the differentially regulated genes identified in the transcriptomic analysis in more detail as they represent an interesting starting point to understand molecular data found for ARD roots and leaves. Furthermore, testing the hypothesis of more efficient detoxification systems in tolerant genotypes may later be used in marker-assisted breeding for more tolerant genotypes.

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4.2. Figures and tables

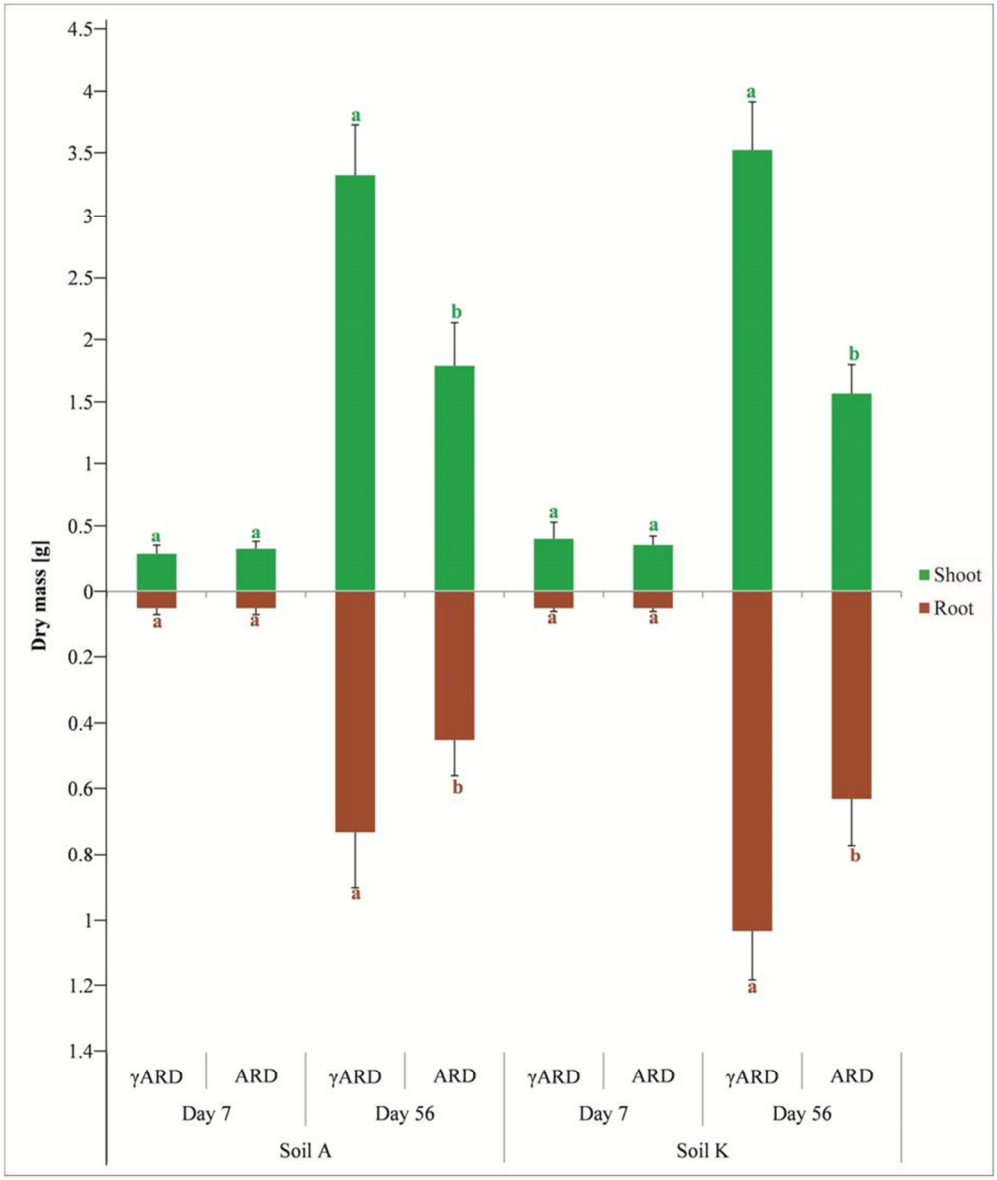


Fig. 1. Per plant dry mass of shoot (green color) and root material (brown color) 7 and 56 days after transfer to γARD or ARD soil of site A or K. Differences between treatments at one time point and soil were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significance indicated by different letters at $P < 0.05$.

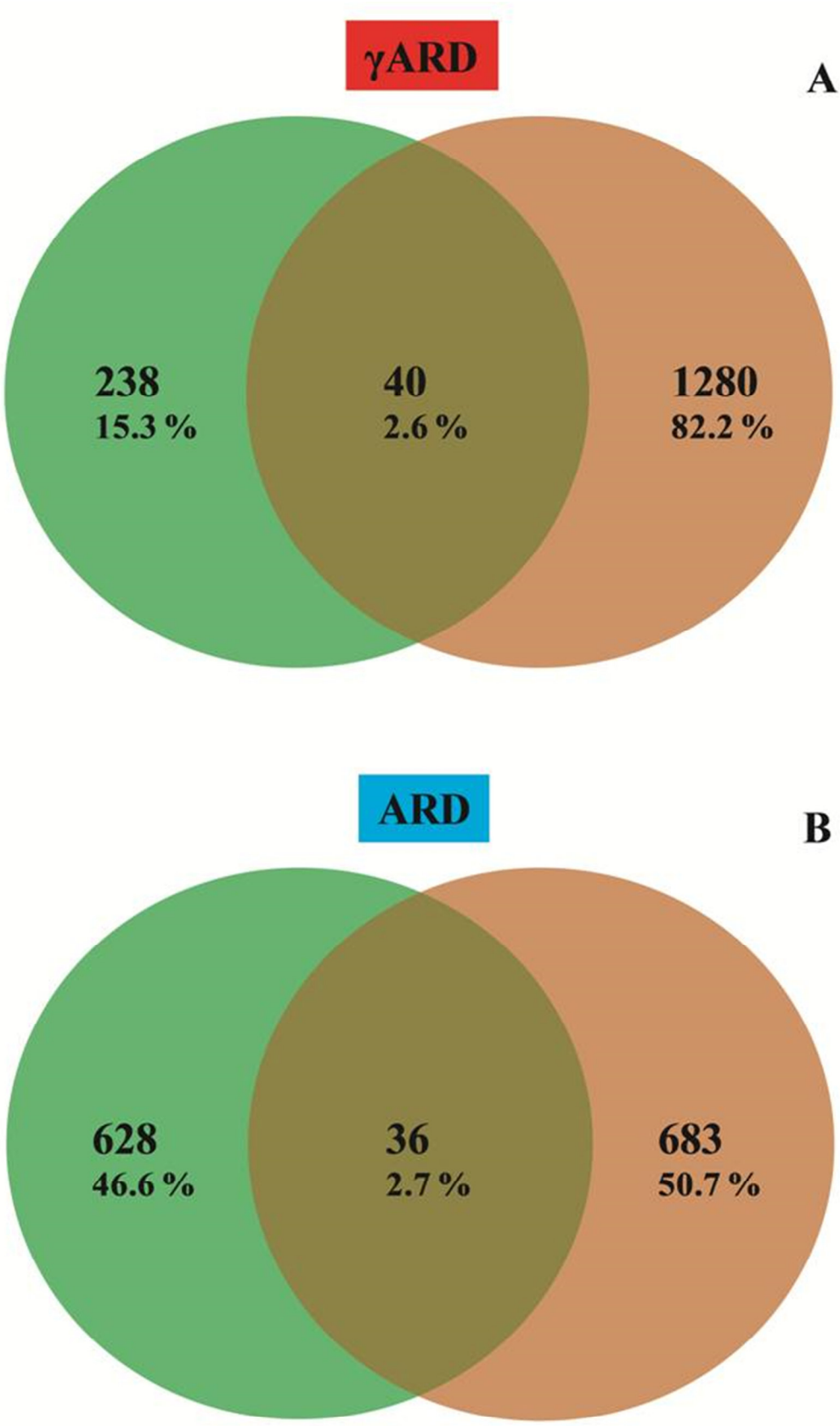


Fig. 2. Venn diagram of genes being exclusively or higher expressed in γARD (A) or ARD samples (B) found in leaves (green color) and roots (brown color) at day 7 of soil A analyzed by MACE (L3P vs. R2P). Genes found to be higher expressed in both tissues (leaves and roots) of one sampling (γARD or ARD) were shared by overlapping circles (olive color). Forty exclusively/higher expressed genes were shared by γARD samples between both tissues whereas ARD samples had 36 mutual exclusively/higher expressed genes present in both tissues. Detailed lists of shared genes are presented in Supplementary Tables S2-3.

Table 1

Primer sequences for gene expression analyses via RT-qPCR.*

Gene name (MDP ID)	Chromosomal location of MDP ID	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
elongation factor 1-alpha (MDP0000304140)	chr11:10,824,588..10,826,029	*EF1a*	f: GAACGGAGATGCTGGTATGG r: CCAGTTGGCTCCTTCTTCTC	159	90.7	0.999
elongation factor 1-beta 2-like (MDP0000903484)	chr2:31,112,380..31,113,206	*EF1b*	f: GAGAGTGGGAAATCCTCTG r: ACCAACAGCAACCAATTTC	138	94.5	0.998
tubulin beta chain (MDP0000951799)	chr4:14,595,834..14,597,758	*TUBB*	f: TTCTCTGGGAGGAGGTACTG r: GTCGCATTGTAAGGCTCAAC	147	93.9	0.999
17.1 kDa class II heat shock protein-like (MDP0000700383)	chr6:5,778,672..5,779,142	*HSP17.1*	f: CGCCGAGAAGTCATTCAAC r: CACGAACACGTAGGAGTTAG	109	98.8	0.999
17.3 kDa class II heat shock protein-like (MDP0000548065)	chr15:46,232,157..46,232,627	*HSP17.3*	f: GACATGCCGGGACTGAAG r: CATTCTCCGGCAACACAAAC	169	90.6	1
18.5 kDa class I heat shock protein-like (MDP0000759666)	chr11:7,597,407..7,597,889	*HSP18.5*	f: GCTTAAGATCAGCGGAGAG r: CTTCGGAACAGTGACACTTAG	175	97.9	0.998
1-aminocyclopropane-1-carboxylate oxidase homolog 1-like (MDP0000314499)	chr1:19,179,184..19,180,712	*ACO1*	f: CGCAGTTGGAGATGAACTTG r: CATGCCGTGATGGACAGTAG	167	107.4	0.996
acidic endochitinase-like (MDP0000218691)	chr1:27,531,001..27,531,894	*CHIA*	f: CTGAGTGCTGACATCAGAAC r: GACTGACCACCTAAGAAGTTG	149	93.6	0.998
ankyrin-1-like (MDP0000157404)	chr15:20,054,312..20,055,259	*ANK1*	f: CAACAAGAATGGCGAAACTC r: CAAGATCAACCACTTCCATAGG	113	93.9	0.999
auxin-responsive protein IAA8-like (MDP0000090281)	chr15:39,374,362..39,376,780	*IAA8*	f: ATCCTGCAAATCAATGAACC r: ACTGATGGCCCTAATCTTCC	176	92.1	0.995
biphenyl synthase 2 (MDP0000716308)	chr15:6,527,251..6,527,937	*BIS2*	f: CTTGTGGTTTGCGCAGAG r: GTCTGCATGCCACGATTTC	172	107	0.993
biphenyl synthase 3 (MDP0000287919)	chr15:6,482,023..6,483,582	*BIS3*	f: GGCAAGAAGCAGCATTGAAAG r: CACAACCTGGCATGTCAAC	105	103.3	0.994
biphenyl synthase 4 (MDP0000302905)	chr14:22,431,166..22,432,738	*BIS4*	f: CAACAAGCAGCACTGAAAGC r: GATGGGTTAAGGCCAAGGAG	140	110.6	0.987
caffeic acid 3-O-methyltransferase-like (MDP0000745475)	chr7:19,754,179..19,755,923	*OMTb*	f: CATCTGAGATCGCAGCTCAC r: GTAGAGCCTCCGGAAACAC	146	103.8	0.997
cytochrome P450 CYP736A12-like (MDP0000205306)	chr11:24,825,865..24,827,637	*B4Ha*	f: GATGTGCACGCTCGAATTAC r: ATTGGCGTCCTCTTGAACAG	106	108	0.998
cytochrome P450 CYP736A12-like (MDP0000152900)	chr11:24,898,086..24,899,852	*B4Hb*	f: GCTGAGTATGGCCCGTATTG r: AGGAACCCGTCGATTATTGG	156	94.1	0.996

Gene name (MDP ID)	Chromosomal location of MDP ID	Abbreviation	Primer sequence (5' - 3')	Fragment length [bp]	Amplification efficiency [%]	Coefficient of determination R^2
disease resistance protein At4g27190-like (MDP0000463166)	chr10:9,979,125..9,981,620	*DRP*	f: GGAGGATCAAGAGCTTAAAC r: GCCTCTTCTACTGTCTCAAC	123	105.9	0.999
ethylene insensitive 3 class transcription factor (EIL2) (MDP0000283197)	chr7:4,202,194..4,204,613	*EIL2*	f: TATTGCCAAGATCCAGAAGC r: GCGAAAGACTCACTCCAAAG	171	104.4	0.996
ethylene insensitive 3 class transcription factor (EIL3) (MDP0000212881)	chr8:29,241,428..29,242,546	*EIL3*	f: GAGCTCGAGAAGAGGATGTG r: CTGTGATTGACGCTGTCTTG	105	97.2	0.999
F-box/kelch-repeat protein At3g06240-like (MDP0000612137)	chr9:9,486,121..9,487,810	*KFB1*	f: GAGCCTATGCACTGGAAATG r: ATCGCCAAGAGAAACAAGG	160	97.4	0.999
gibberellin-regulated protein 1-like (MDP0000140078)	chr12:21,219,568..21,220,198	*GASA1*	f: CGTTGCAGCTGTGTTCCTC r: CATCTGCATGCCCGAATATGAG	156	104.3	0.998
LRR receptor-like serine/threonine-protein kinase MRH1 (MDP0000302779)	chr10:27,382,369..27,386,559	*MRH1*	f: CGAGGTTTCATGGTTGTTG r: CGCAGAATGACAACAGAATC	163	92.7	0.989
pathogenesis-related genes transcriptional activator PTI5-like (MDP0000805422)	chr5:4,859,770..4,860,342	*PTI5*	f: AGCTGAAATCCGTGACTCTG r: TAAGCATGGCCTTAGTACCG	128	91.2	0.998
senescence-associated carboxylesterase 101-like (MDP0000837935)	chr1:10,138,107..10,139,279	*SAG101*	f: CGTAAACTAGCGATGCAGAAG r: GGACTCCTCCTTGTACCATTC	102	94.5	0.998
thaumatin-like protein 1a (MDP0000552328)	chr9:28,418,285..28,419,411	*TL1*	f: ACGGCTTCAACTTGCCTATG r: GCCGCTTTCACTTGAAGTTG	118	91.9	0.998
thaumatin-like protein 1b (MDP0000782642)	chr9:14,576,503..14,577,513	*TL1b*	f: GTCCCTAACCTCCGATCAAC r: GTGGCACAACTGAACTTTCC	159	96.1	0.999
wound-induced protein 1-like (MDP0000792101)	chr12:25,381,423..25,381,845	*WIN1*	f: GATTCCAGCCACACTGACTTC r: CTCCCTGAACTGAGTGATCAAC	150	92.5	0.997
zinc finger protein ZAT12 (MDP0000595671)	chr3:8,838,087..8,838,608	*ZAT12*	f: CAAGAAGCCGAAGCTAATGC r: GTCCGACCGTGAACTCAAG	125	94.9	0.972

*All primers (f: forward, r: reverse) were evaluated in an amplification efficiency test at an annealing temperature of 60 °C. Bold printing indicates reference genes.

Table 2

Identified nutrients [mg/g dry mass] in shoot material of γARD and ARD samples at day 7 and 56 in soil A.*

Nutrient	Day 7		Day 56	
	γARD	ARD	γARD	ARD
Al	0.28 ± 0.08	0.27 ± 0.11	0.12 ± 0.09	0.19 ± 0.2
B	0.02 ± 0	0.02 ± 0	0.03 ± 0*	0.02 ± 0
Ba	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
C	28.76 ± 3.97	27.75 ± 2.88	27.48 ± 1.18*	25.38 ± 1.33
Ca	7.55 ± 1.33	8.24 ± 0.54	5.43 ± 0.64**	7.37 ± 0.53
Fe	0.52 ± 0.17	0.43 ± 0.19	0.17 ± 0.11	0.3 ± 0.31
K	10.38 ± 1.07	9.02 ± 0.96	14.08 ± 4.09	11.89 ± 2.38
Mg	1.68 ± 0.18*	1.96 ± 0.14	1.54 ± 0.05	1.45 ± 0.12
Mn	0.08 ± 0.02	0.06 ± 0.01	0.21 ± 0.02	0.24 ± 0.03
N	464.08 ± 4.18	465.66 ± 3	476.32 ± 7.7	474.39 ± 4.01
Na	0.81 ± 0.17*	1.18 ± 0.18	0.77 ± 0.66	0.45 ± 0.23
P	2.52 ± 0.25	2.58 ± 0.24	0.97 ± 0.08*	0.83 ± 0.07
S	3.23 ± 0.3*	3.86 ± 0.37	2.83 ± 0.12	2.75 ± 0.36
Sr	0.03 ± 0.01	0.03 ± 0	0.02 ± 0***	0.04 ± 0
Zn	0.05 ± 0.02	0.05 ± 0.02	0.08 ± 0.12	0.05 ± 0.01

*Differences between treatments at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Table 3

Comparison of gene expression analyzed by RT-qPCR in leaves of plants cultivated in soil coming from different sites (soil A and soil K) at day 7 linked to root data.*

Class	Gene Name	Leaves expression γARD ± SD soil A	Leaves expression ARD ± SD soil A	Leaves expression γARD ± SD soil K	Leaves expression ARD ± SD soil K	Leaves ratio (ARD/γARD) soil A	Leaves ratio (ARD/γARD) soil K	Roots ratio (ARD/γARD) soil A	Roots ratio (ARD/γARD) soil K
Phytoalexin biosynthesis	*BIS2*	0 ± 0	0.01 ± 0.02	0 ± 0	0 ± 0	8.68	2.31	3.67*	18.5***
Phytoalexin biosynthesis	*BIS3*	0.01 ± 0	0.03 ± 0.03	0 ± 0	0 ± 0	4.12	1.93	4.11**	10.58***
Phytoalexin biosynthesis	*BIS4*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.57	1.47	3.9*	23.95***
Phytoalexin biosynthesis	*OMTb*	0 ± 0	0.01 ± 0.01	0 ± 0	0 ± 0	2.9	1.03	2.05**	2.96***
Phytoalexin biosynthesis	*B4Ha*	0 ± 0	0.01 ± 0.01	0 ± 0	0 ± 0	2.84	1.43	2.78**	5.62***
Phytoalexin biosynthesis	*B4Hb*	0.05 ± 0.03	0.1 ± 0.1	0.01 ± 0	0.02 ± 0.01	2.13	1.54	2.61**	5.83***
Plant defense	*TL1*	0.6 ± 0.37	1.87 ± 2.11	0.2 ± 0.05	0.14 ± 0.03	3.09	-1.36	4.32**	4.88*
Plant defense	*TL1b*	0.63 ± 0.17	1.36 ± 1.65	0.8 ± 0.25	0.97 ± 0.18	2.14	1.21	n. a.	n. a.
Regulatory function	*HSP17.1*	1.07 ± 0.84	0.25 ± 0.24	0.01 ± 0	0.01 ± 0	-4.23*	1.08	-3.49***	1.11
Regulatory function	*HSP17.3*	14.02 ± 7.54	1.39 ± 1.01	0.06 ± 0.02	0.04 ± 0.01	-10.12**	-1.5	-3.37***	1.72*
Regulatory function	*HSP18.5*	1.19 ± 0.56	0.11 ± 0.03	0.03 ± 0	0.03 ± 0	-11.16***	-1.06	-3.9***	1.13
Regulatory function	*KFB1*	0.03 ± 0	0.03 ± 0.01	0.01 ± 0	0.01 ± 0	-1.02	1.27	n. a.	n. a.
Signaling	*EIL2*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.21	1.16	n. a.	n. a.
Signaling	*EIL3*	1.48 ± 0.31	1.49 ± 0.46	1.25 ± 0.31	1.43 ± 0.21	1	1.14	n. a.	n. a.
Signaling	*IAA8*	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0	0.02 ± 0	-1.05	-1	n. a.	n. a.
Signaling	*PTI5*	0.1 ± 0.04	0.19 ± 0.14	0.03 ± 0.01	0.04 ± 0.01	1.93	1.3	n. a.	n. a.

*For normalization *EF1a*, *EF1b* and *TUBB* were used as reference genes. Ratios are represented by the quotient of ARD/γARD with values between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Differences between treatments were tested using a Welch Two Sample t-test (n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Correlation between MACE data and RT-qPCR of soil A was $R = 0.83$ and $P < 0.001$, correlation between leaf RT-qPCR data of soil A and soil K was $R = 0.65$ and $P < 0.01$. Ratio of roots was obtained from Weiß et al. (in preparation).

Table 4

Sequencing results of the MACE analysis for γARD and ARD samples of soil A.*

Sample	Number of raw reads	Average length before trimming [bp]	Reads after trimming	Average length after trimming [bp]	Non-specifically mapped reads [number | % trimmed reads]		Unique mapped reads [number | % trimmed reads]	
γARD 1	12,123,247	109.2	12,123,114	109.1	211,491	[1.74]	3,640,907	[30.03]
γARD 2	12,413,200	110.1	12,413,056	110	222,797	[1.79]	3,790,006	[30.53]
γARD 3	13,519,267	109.9	13,519,110	109.8	250,502	[1.85]	4,212,351	[31.16]
ARD 1	14,403,565	109.4	14,403,387	109.3	237,572	[1.65]	4,423,593	[30.71]
ARD 2	10,576,129	109.7	10,576,001	109.6	167,421	[1.58]	3,265,082	[30.87]
ARD 3	9,450,844	110	9,450,741	109.9	145,234	[1.54]	2,882,859	[30.5]

*Sequences were trimmed employing poly-A adapter sequences using CLC Genomics Workbench 8. Trimmed sequences were mapped to the predicted transcriptome of *Malus domestica*. Reads mapped to more than one sequence (non-specifically mapped reads) were not included in further analyses.

Table 5

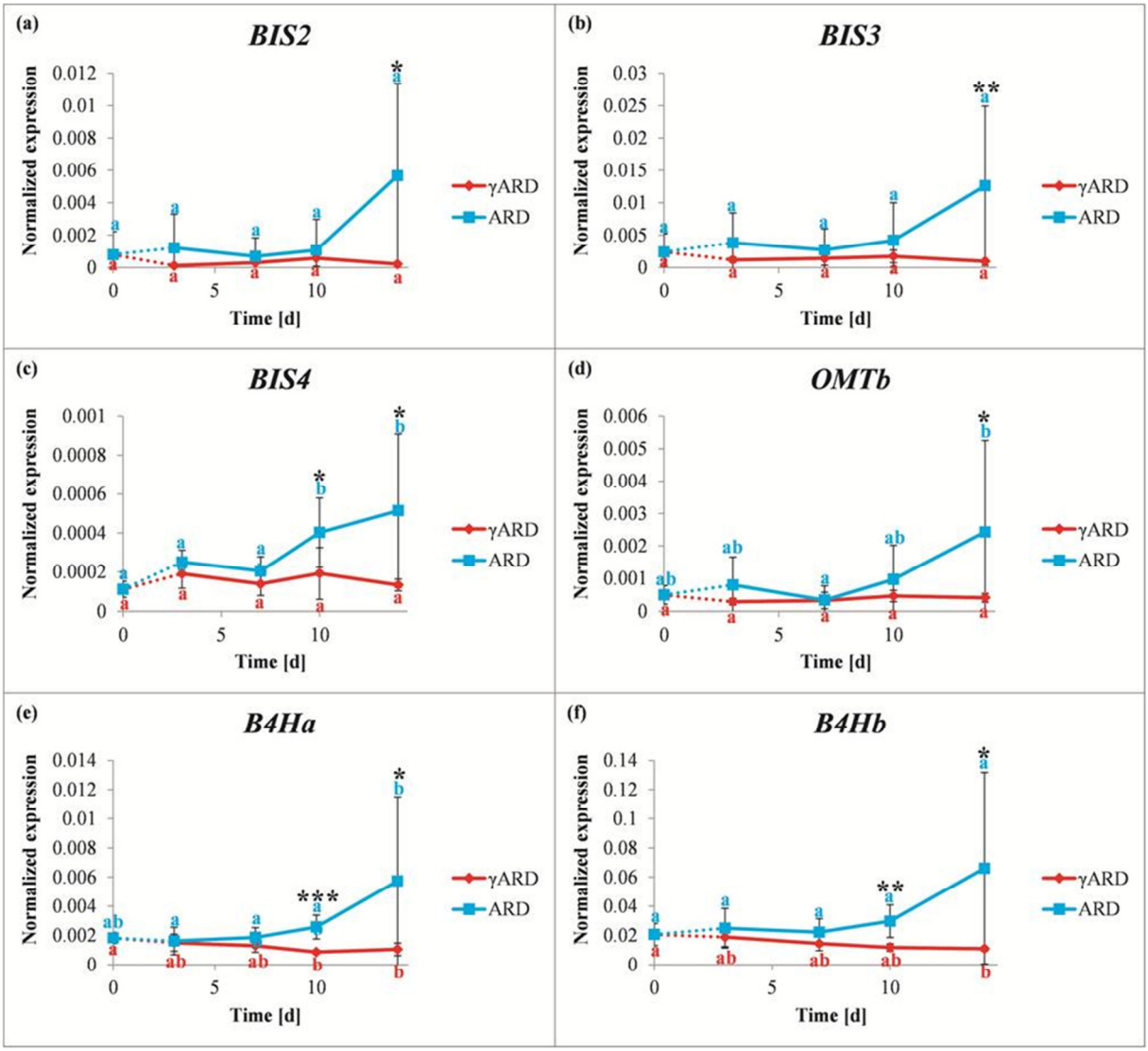
List of genes with a FDR corrected *P*-value < 0.05, a contribution value > 0.69 % and at least 2-fold regulation or which were found only in either γARD or ARD samples.*

MapMan functional category	Gene	Chromosomal location of MDP ID	Normalized transcript abundance γARD ± SD	Normalized transcript abundance ARD ± SD	Ratio	Contribution [%]
DNA processing	DNA topoisomerase 2-like (MDP0000211904)	chr2:35,266,329..35,269,618	0.54 ± 0.21	0.05 ± 0.08	-11.76	2.34
Lipid metabolism	phospholipase A1-IIdelta-like (MDP0000127930)	chr17:23,168,930..23,170,246	0.03 ± 0.05	0 ± 0	only γARD	2.22
Miscellaneous	anthocyanidin 3-O-glucosyltransferase 5-like (MDP0000244946)	chr2:13,399,641..13,400,828	0.03 ± 0.05	0 ± 0	only γARD	2.54
Miscellaneous	**GDSL esterase/lipase 5-like (MDP0000244607)**	**chr16:5,786,287..5,791,158**	**1.91 ± 0.51**	**3.96 ± 0.62**	**2.08**	**0.73**
Miscellaneous	caffeic acid 3-O-methyltransferase-like (MDP0000657537)	chr1:28,723,194..28,724,542	0.36 ± 0.18	1 ± 0.35	2.76	0.79
Miscellaneous	UDP-glucose flavonoid 3-O-glucosyltransferase 7-like (MDP0000012541)	chr7:669,358..670,773	0.02 ± 0.04	0.37 ± 0.14	14.93	2.28
Not assigned	protein RETICULATA-RELATED 1 (MDP0000571356)	chr8:28,168,746..28,173,436	2.38 ± 0.49	1.1 ± 0.6	-2.16	0.87
Not assigned	**translation initiation factor IF-3-like (MDP0000337845)**	**chr2:1,373,124..1,373,517**	**1.23 ± 0.44**	**2.7 ± 0.45**	**2.20**	**1.15**
Not assigned	**mechanosensitive ion channel protein 1 (MDP0000359809)**	**chr11:27,273,243..27,273,378**	**0.71 ± 0.16**	**1.59 ± 0.31**	**2.23**	**0.83**
Not assigned	**ABA-inducible protein PHV A1-like (MDP0000429218)**	**chr5:4,538,731..4,539,552**	**0.61 ± 0.24**	**1.53 ± 0.34**	**2.52**	**0.93**
Not assigned	putative UPF0481 protein At3g02645 (MDP0000161332)	chr5:19,075,194..19,076,876	0.45 ± 0.21	1.18 ± 0.55	2.60	0.97
Not assigned	transcription factor PRE6-like (MDP0000174388)	chr16:3,281,914..3,282,592	0.1 ± 0.09	0.59 ± 0.04	5.69	2.29
Not assigned	**cyclin-D5-1-like (MDP0000213677)**	**chr8:12,108,023..12,108,651**	**0 ± 0**	**0.29 ± 0.19**	**only ARD**	**1.09**
Protein processing	elongation factor 1-gamma-like (MDP0000701209)	chr15:45,892,060..45,892,900	0.26 ± 0.03	0.52 ± 0.21	2.01	0.75
Protein processing	**AAA-ATPase ASD, mitochondrial-like (MDP0000676693)**	**chr17:2,924,006..2,926,952**	**1.14 ± 0.35**	**2.77 ± 1.18**	**2.44**	**0.76**
Protein processing	**mechanosensitive ion channel protein 6 (MDP0000748642)**	**chr10:7,042,502..7,055,407**	**0.16 ± 0.08**	**0.55 ± 0.15**	**3.44**	**0.80**
Protein processing	**ATP-dependent zinc metalloprotease FTSH 9 (MDP0000468286)**	**chr11:7,083,368..7,088,664**	**0.16 ± 0.08**	**0.63 ± 0.29**	**3.92**	**0.97**
Protein processing	**F-box protein SKIP2-like (MDP0000415972)**	**chr8:13,916,191..13,917,105**	**0 ± 0**	**0.06 ± 0.05**	**only ARD**	**1.19**
RNA processing	heat shock factor protein HSF30-like (MDP0000489886)	chr8:8,198,430..8,199,827	1.22 ± 0.5	0 ± 0	only γARD	2.45
RNA processing	**transcription factor HEC2-like (MDP0000249405)**	**chr15:2,402,089..2,402,841**	**0.73 ± 0.28**	**1.51 ± 0.17**	**2.08**	**1.13**
RNA processing	**two-component response regulator ORR9-like (MDP0000585643)**	**chr10:12,676,869..12,683,728**	**0.98 ± 0.27**	**2.37 ± 0.59**	**2.43**	**0.76**
RNA processing	ethylene-responsive transcription factor ERF113 (MDP0000316694)	chr4:7,197,134..7,203,922	0.48 ± 0.15	1.24 ± 0.5	2.60	0.72
RNA processing	**major allergen Mal d 1-like (MDP0000116244)**	**chr16:11,433,701..11,434,305**	**0.34 ± 0.19**	**1.04 ± 0.41**	**3.01**	**1.08**
RNA processing	probable WRKY transcription factor 9 (MDP0000137704)	chr15:43,865,669..43,869,894	0.11 ± 0.09	0.62 ± 0.1	5.71	1.98
RNA processing	major allergen Mal d 1-like (MDP0000260110)	chr13:14,514,476..14,514,955	0.02 ± 0.04	0.37 ± 0.24	14.89	2.40

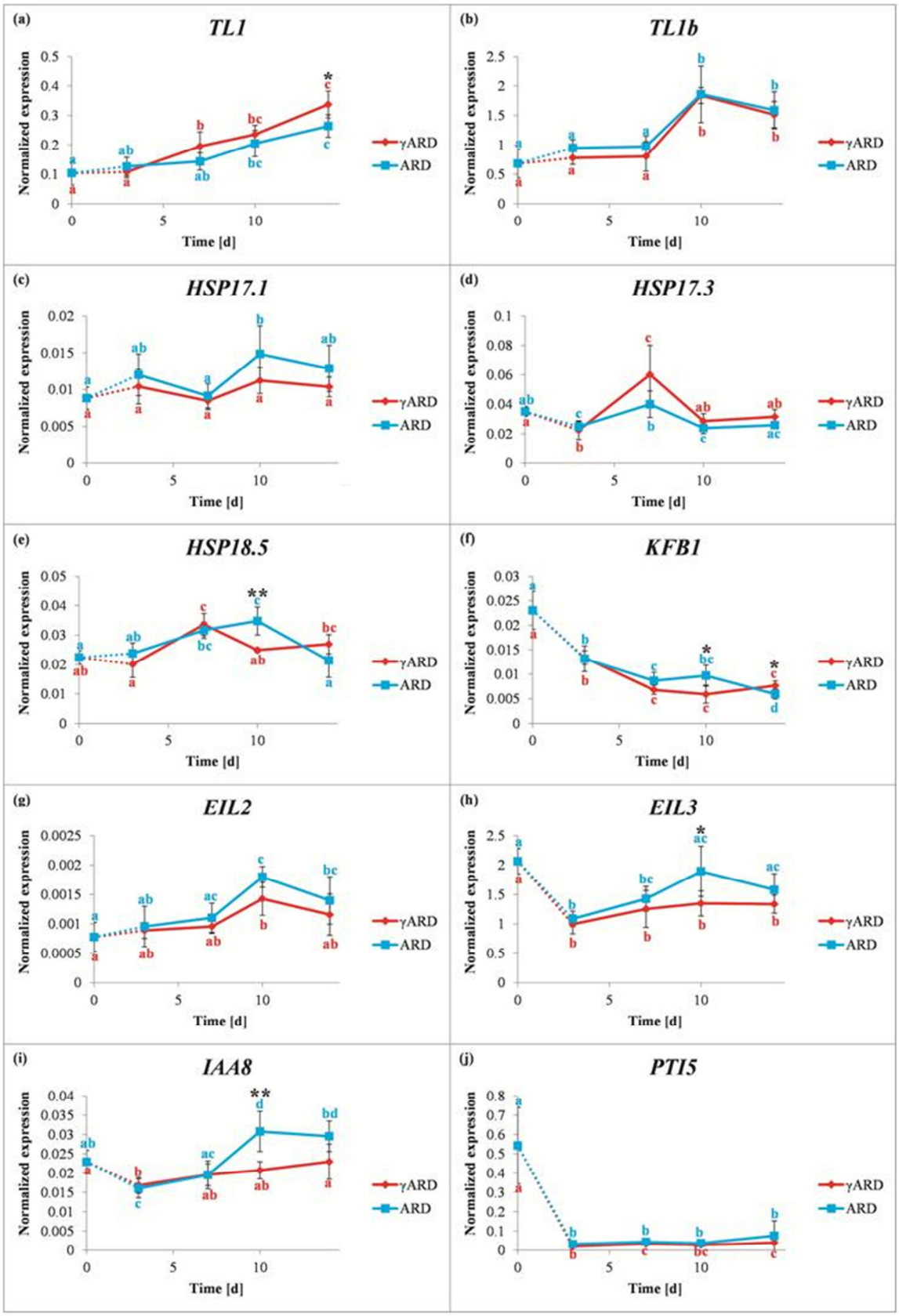
MapMan functional category	Gene	Chromosomal location of MDP ID	Normalized transcript abundance γARD ± SD	Normalized transcript abundance ARD ± SD	Ratio	Contribution [%]
Signaling	**probable calcium-binding protein CML27 (MDP0000330029)**	**chr14:28,314,468..28,315,041**	**0.91 ± 0.24**	**1.92 ± 0.72**	**2.11**	**0.72**
Signaling	**cysteine-rich receptor-like protein kinase 10 (MDP0000121305)**	**chr6:23,096,149..23,100,640**	**1.66 ± 0.54**	**4.33 ± 0.63**	**2.60**	**0.70**
Signaling	**high-affinity nitrate transporter 3.2 (MDP0000277926)**	**chr6:19,059,166..19,060,729**	**0.49 ± 0.35**	**1.82 ± 0.49**	**3.74**	**0.77**
Stress	heat shock protein 90-2-like (MDP0000853638)	chr9:238,476..238,913	0.72 ± 0.24	0.2 ± 0.17	-3.59	1.87
Stress	putative disease resistance protein RGA3 (MDP0000142781)	chr8:12,817,214..12,820,123	0.02 ± 0.04	0.06 ± 0.05	2.37	1.78

*Ratios are represented by the quotient of ARD/γARD with values between 0 and 1 being transformed by using the negative reciprocal value of the quotient. Genes are ordered in alphabetical order according to their MapMan functional category and their ratio from lowest to highest value. Contribution gives the value of the gene in the PCA where a value > 0.69 % is considered relevant for contribution in separating the groups (ANOSIM: $R = 1$, $P = 0.1$). Bold printing indicates significant correlation ($P < 0.05$) to the shoot length according to **Supplementary Fig. S3**.

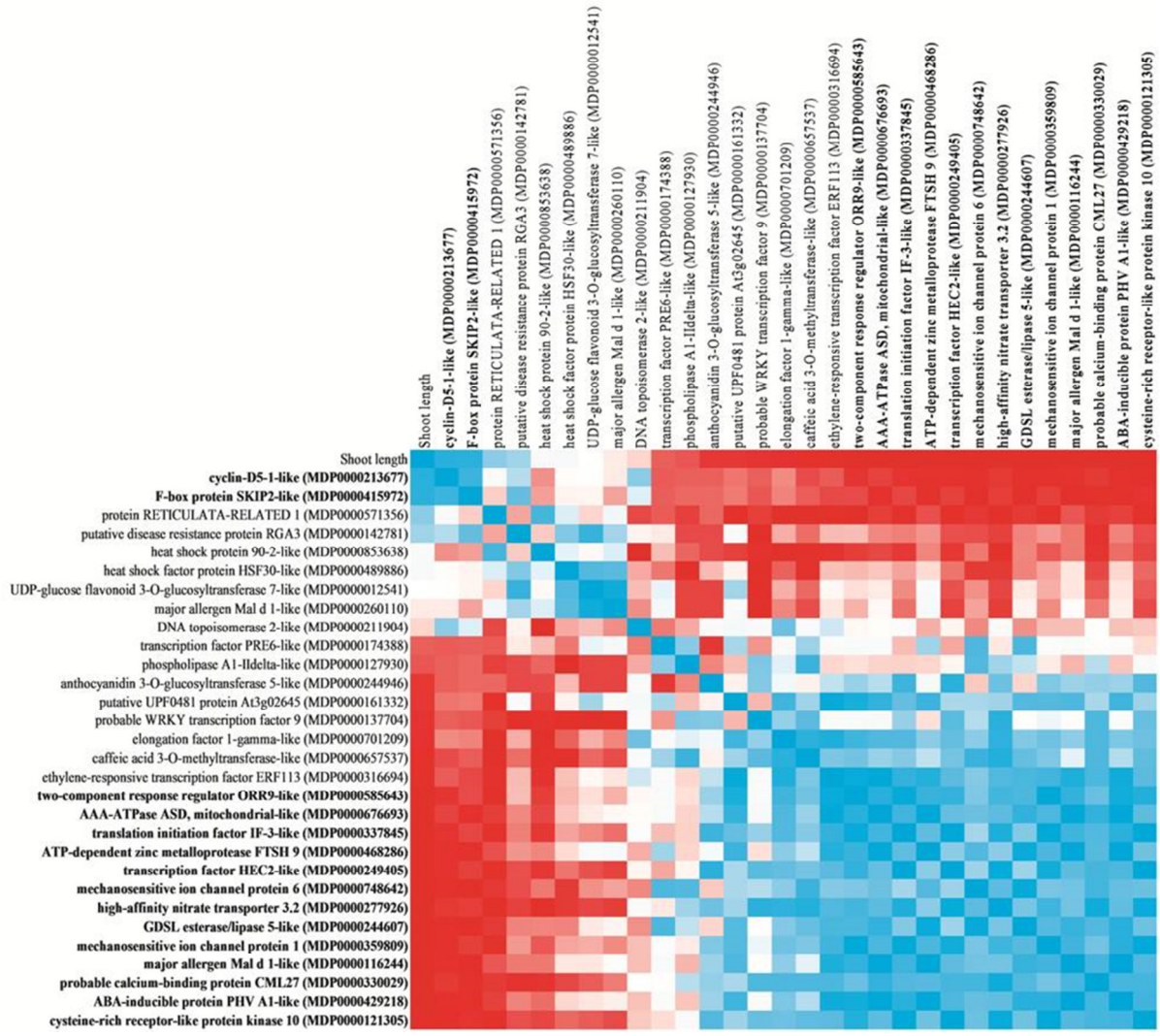
4.3. Supplementary data



Supplementary Fig. S1. Expression of phytoalexin biosynthesis genes in γARD and ARD leaf samples analyzed by RT-qPCR. Differences between treatments for *BIS2* (a), *BIS3* (b), *BIS4* (c), *OMTb* (d), *B4Ha* (e) and *B4Hb* (f) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were tested with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD). The connection from the start of the experiment and day 3 is indicated by a dotted line, because the plant material at day 0 represented freshly uprooted acclimatized plantlets which can only be partly compared to the freshly potted plantlets at day 3.



Supplementary Fig. S2. Expression of plant defense genes, genes with regulatory functions and signaling genes in γARD and ARD leaf samples analyzed by RT-qPCR. Differences between treatments for *TL1* (a), *TL1b* (b), *HSP17.1* (c), *HSP17.3* (d), *HSP18.5* (e), *KFB1* (f), *EIL2* (g), *EIL3* (h), *IAA8* (i) and *PTI5* (j) at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were tested with different letters indicating significant differences ($P < 0.05$, red color for γARD, blue color for ARD). The connection from the start of the experiment and day 3 is indicated by a dotted line, because the plant material at day 0 represented freshly uprooted acclimatized plantlets which can only be partly compared to the freshly potted plantlets at day 3.



Supplementary Fig. S3. Correlation matrix of shoot length and gene expression in leaf samples at day 7 in soil A. Blue color indicates a positive correlation between variables whereas red color stands for a negative correlation between variables. The intensity of the color shows the level of correlation between variables with darker colors representing stronger correlations (n = 3). Diagonally, values equal X = 1 as the variables represent a correlation with themselves plus along this diagonal axis values are mirrored. Genes were ordered from top to bottom according to their correlation with the shoot length. Bold printing indicates significant correlations among shoot length and genes.

Supplementary Table S1

Shoot growth response given as shoot length of 'M26' plants to γARD and ARD soil of two different sites (A and K).*

Time	Site A		Site K	
	γARD [cm]	ARD [cm]	γARD [cm]	ARD [cm]
Day 0	3 ± 0.3 a	2.6 ± 0.3 a	4.3 ± 1.1 a	4.4 ± 1.1 a
Day 3	n. a.	n. a.	4.5 ± 0.8 a	5.1 ± 1.2 abc
Day 7	3 ± 0.4 a	2.7 ± 0.3 a	5.7 ± 1.1 *** b	5 ± 1.1 b
Day 10	n. a.	n. a.	6.5 ± 1.1 ** c	5.6 ± 0.8 cd
Day 14	3.8 ± 0.5 *a	2.9 ± 0.4 a	6.6 ± 1.3 *** c	5.5 ± 1.2 c
Day 21	5 ± 0.6 ***b	3.2 ± 0.5 a	7.1 ± 1.6 *** c	5.4 ± 1.1 bc
Day 28	10 ± 0.6 ***c	6.1 ± 0.3 b	9.2 ± 2.1 *** d	5.6 ± 0.9 cd
Day 35	13.4 ± 1 ***d	8.2 ± 0.5 bc	11.3 ± 2.8 *** de	6 ± 0.9 cde
Day 42	17.2 ± 1.3 ***de	10.5 ± 1 cd	13.9 ± 3.8 *** ef	6.3 ± 1 df
Day 49	22 ± 1.4 ***e	12.8 ± 1.8 de	16.6 ± 4.6 *** fg	6.6 ± 1.6 ef
Day 56	28.3 ± 2.3 ***f	15.8 ± 2.3 e	19.9 ± 5.3 *** g	7.9 ± 2.7 f

*Differences between treatments at one time point were tested using a Welch Two Sample t-test (means and standard deviations of n = 5 for soil A and $n_0 = 175$, $n_{3;10;21;28;35;42;49;56} = 35$, $n_7 = 140$, $n_{14} = 70$ for soil K) with significant differences shown for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Using a Tukey multiple comparisons test, differences between time points within one treatment were tested with different letters indicating significant differences ($P < 0.05$). n. a. not available.

Supplementary Table S2

List of genes shared among leaves and roots with higher expression in γARD samples.*

MapMan functional category	MDP ID	Name
Hormone metabolism	**MDP0000565846**	**multiprotein-bridging factor 1c-like**
Hormone metabolism	**MDP0000303198**	**multiprotein-bridging factor 1c-like**
Major CHO metabolism	MDP0000285388	16.6 kDa heat shock protein-like
Not assigned	**MDP0000150261**	**conserved oligomeric Golgi complex subunit 7**
Not assigned	**MDP0000849143**	**desumoylating isopeptidase 1**
Not assigned	MDP0000192271	molybdate-anion transporter-like
Not assigned	MDP0000652797	pentatricopeptide repeat-containing protein At3g16010-like
Not assigned	MDP0000247769	protein EXECUTER 1, chloroplastic-like
Not assigned	**MDP0000657396**	**protein FAM32A-like**
Protein processing	MDP0000190008	10 kDa chaperonin-like
Protein processing	MDP0000336201	CDPK-related kinase 3-like
Protein processing	MDP0000142577	subtilisin-like protease SBT2.2
Protein processing	**MDP0000570186**	**ubiquitin-like protein 5**
RNA processing	**MDP0000243895**	**heat shock factor protein HSF30**
RNA processing	MDP0000155667	heat shock transcription factor 4 (HSF4)
RNA processing	MDP0000174161	heat stress transcription factor A-3-like
RNA processing	MDP0000228898	protein S-acyltransferase 1
Stress	MDP0000291831	17.1 kDa class II heat shock protein-like
Stress	MDP0000700383	17.1 kDa class II heat shock protein-like
Stress	**MDP0000548065**	**17.3 kDa class II heat shock protein-like**
Stress	**MDP0000621193**	**17.3 kDa class II heat shock protein-like**
Stress	MDP0000172108	18.1 kDa class I heat shock protein-like
Stress	**MDP0000759666**	**18.5 kDa class I heat shock protein**
Stress	**MDP0000795157**	**dnaJ homolog subfamily B member 1-like**
Stress	**MDP0000254260**	**heat shock protein 83-like**
Stress	MDP0000161691	hsp70-Hsp90 organizing protein 3-like
Stress	MDP0000145097	MLO-like protein 11
Stress	MDP0000214382	small heat shock protein
Transport	MDP0000280043	cation/H(+) antiporter 15-like
Transport	MDP0000173864	TMV resistance protein N-like

*List corresponds to **Fig. 2**. Genes are ordered in alphabetical order according to their MapMan functional category. Bold printing indicates genes also part of the FTP data set (**Supplementary Table S4**).

Supplementary Table S3

List of genes shared among leaves and roots with higher expression in ARD samples.*

MapMan functional category	MDP ID	Name
Development	MDP0000279018	late embryogenesis abundant protein At1g64065-like
Hormone metabolism	MDP0000127134	ethylene-responsive transcription factor 1B-like
Hormone metabolism	MDP0000229843	protein DMR6-LIKE OXYGENASE 1-like
Lipid metabolism	MDP0000537488	phospholipid:diacylglycerol acyltransferase 1-like
Metal handling	MDP0000412490	nicotianamine synthase-like
Miscellaneous	MDP0000318256	(R)-mandelonitrile lyase 3-like
Miscellaneous	MDP0000204569	blue copper protein-like
Miscellaneous	MDP0000152900	cytochrome P450 CYP736A12-like
Miscellaneous	MDP0000247130	glucan endo-1,3-beta-glucosidase-like
Miscellaneous	**MDP0000511650**	**glutathione S-transferase-like**
N-metabolism	**MDP0000585462**	**nitrate reductase [NADH]**
Not assigned	MDP0000162146	desiccation-related protein PCC13-62-like
Not assigned	MDP0000732061	molybdate transporter 1
Not assigned	MDP0000346805	pentatricopeptide repeat-containing protein At4g35850
Not assigned	MDP0000374466	protein FLX-like 2
Protein processing	MDP0000233037	CBL-interacting serine/threonine-protein kinase 4-like
Protein processing	MDP0000619285	F-box/kelch-repeat protein At5g43190-like
RNA processing	MDP0000286430	heterogeneous nuclear ribonucleoprotein 1-like
RNA processing	MDP0000253189	probable WRKY transcription factor 51
Secondary metabolism	MDP0000716308	biphenyl synthase 2
Signaling	MDP0000174381	G-type lectin S-receptor-like serine/threonine-protein kinase LECRK3
Signaling	MDP0000172803	receptor-like protein kinase HAIKU2
Stress	MDP0000218691	acidic endochitinase-like
Stress	MDP0000888042	acidic endochitinase-like
Stress	MDP0000280265	acidic endochitinase-like
Stress	MDP0000552328	thaumatin-like protein 1a
Stress	MDP0000782642	thaumatin-like protein 1b

*List corresponds to **Fig. 2**. Genes are ordered in alphabetical order according to their MapMan functional category. Bold printing indicates genes also part of the FTP data set (**Supplementary Table S4**).

For Supplementary Table S4 see disk in the back of the thesis

Supplementary_Table_S4_Manuscript_III_Chapter_4.pdf

5. General discussion

ARD is not well understood on the molecular level in planta. This work aimed to contribute to uncover involved mechanisms in the disease etiology. The results of the three submitted studies were already covered and discussed in the manuscripts. Here, main findings will be summarized and additional aspects will be emphasized based on the objectives (see *1.9.*). Furthermore, a conclusion and outlook on future research based on these studies will be given.

5.1. How do ARD affected 'M26' plants react to ARD on the transcriptomic level?

Unraveling the responses of ARD affected 'M26' plants on the transcriptomic level was the main driver of this study (see **2.**, **3.** and **4.**). The use of MACE and coupled RT-qPCR analyses resulted for the first time in an overview of molecular reactions in the highly ARD-sensitive apple rootstock 'M26'. Taking all the findings into consideration it is justified in saying that strong biotic stress responses take place on the transcriptomic level in both root and leaf material but the reaction towards ARD was not adequate as ARD-challenged plants still demonstrated severe growth inhibition. The growth inhibition was incited by biotic factors in ARD soils because disinfection improved plant growth tremendously. Most interestingly, PA biosynthesis genes and corresponding products were found to be differentially regulated and their possible role in ARD will be further discussed (see *5.5.*). Next to the obvious lack of effect from PAs, surprisingly, the often reported function of phytohormones in biotic stress responses was also potentially suppressed or impaired in this study based on gene expression data, and first experiments dealing with the quantification of jasmonic acid (JA) in roots did not reveal differences between variants either (group of Prof. Winkelmann, Hannover, unpublished data). Next to JA, ethylene (ET) usually plays an important role in defense responses but in this study diverse behavior of ET genes were detected, therefore future studies would have to test more genes in more detail to make definitive conclusions about the role of ET, JA and other phytohormones in ARD responses. Nevertheless, the role of phytohormones in root-shoot signaling may have been affected as Shin et al. (2016) reported about the possibly unique role of cytokinin in ARD, and the upregulation of a two-component response regulator involved in the cytokinin-activated signaling pathway was higher expressed in ARD samples of this study as well (see **4.**) which presents an interesting starting point for additional gene expression analyses including more cytokinin related genes. Furthermore, root-shoot signaling may have been hinted at by the upregulation of a high-affinity nitrate transporter involved in NO signaling as discussed before (see **4.**). In sum, 'M26' plants possibly reacted with inefficient defense reactions to ARD causal agents and defense responses presumably also may have been suppressed by the biotic stressors. Functional analysis of discussed genes may help in further understanding affected molecular reactions in ARD-challenged 'M26' plants, in particular root-shoot communication seems to present an interesting starting point for future experiments as roots are in direct contact with the biotic stress but shoots show first observable ARD symptoms in the form of diminished shoot growth.

5.2. Does biotic stress by exposing the root system to ARD lead to a systemic response in aboveground tissue?

Roots are in direct contact with the soil and therefore have to endure the biotic stress presumably in form of microorganisms located in the rhizosphere continuously. Nevertheless, first striking symptoms of ARD can be observed aboveground as shoots will be diminished in their potential growth whereas roots show a delayed effect regarding biomass. Yim et al. (2013) stated that root dry mass was not as severely affected by ARD as shoot dry mass because roots needed to grow more to counterbalance damages in the root system. Hence, in regard of the actual effect of ARD, plants react to the problem with a systemic response. Based on the results obtained in this work, it may be even concluded that also on the transcriptomic level there is a systemic response to biotic stress as outlined by the simultaneous expression of many – albeit differing – biotic stress associated genes. However, candidate genes – identified in roots – analyzed in this study did not show a systemic response and in general only a small portion of genes were simultaneously expressed in both tissues (see **4.**). Only PA biosynthesis genes showed a delayed systemic response to ARD. Genes of the *BIS*, *OMT* and *B4H* gene families revealed an upregulation in ARD variants starting from days 10-14. However, compared to their counterparts in roots, the expression was extremely low. Based on the transcriptomic analysis it can be concluded that plants possibly suffered from systemic oxidative stress as demonstrated by the upregulation of many genes involved in ROS induced reactions both in roots and leaves. These results are in line with observations in ARD affected apple seedlings where ARD led to systemic oxidative stress (Henfrey et al. 2015).

5.3. Are ARD affected molecular reactions in the plant conserved among different ARD soils?

It was of utmost interest if identified genes showed a similar behavior in 'M26' plants grown on ARD soils differing in cropping history and soil properties because next to better understanding ARD on a molecular level in planta, genes with conserved expression among different soils could be used as indicator genes for using the highly susceptible apple rootstock 'M26' in testing soils for ARD incidence which would represent a further improvement of the biotest system established by Yim et al. (2013). Possible candidates for indicator genes have to be stably expressed in ARD-affected plants even when faced with different ARD soils and they should show a strong and early expression for easy detection via RT-qPCR techniques.

In this work, it could be shown that the employed ARD soils differed in ARD severity which was shown by different shoot growth rates in soil A and soil K, and the conserved but higher regulation of PA biosynthesis genes in roots of ARD variants cultivated in soil K (see **3.**). Especially, *BIS* genes showed the highest expression led by *BIS3* which may be used as a potential indicator gene. But potential indicator genes will have to be tested in additional different ARD soils to collect more data points. Nevertheless, the very early induction of PA biosynthesis genes in 'M26' is a promising result for using these candidates as indicators of ARD severity in soils. However, not all genes were

conserved in their expression in 'M26' among different soils, e.g. heat shock protein genes, hinting at soil specific reactions induced by diverse biotic stressors in ARD soils or at indeed heat stress.

5.4. Is there a time-dependent effect on ARD affected molecular reactions?

A time-dependent expression of candidate genes could be observed but was also dependent on the candidate gene as well as tissue analyzed (see **3.** and **4.**). The most impressive results were once again obtained for PA biosynthesis genes which showed a very high expression and regulation already after 3 days in roots. Whereas *BIS* genes stayed on the same expression level afterwards, *OMT* and *B4H* revealed an additional peak after 10 days. As the actual products, biphenyls and dibenzofurans. were found in elevated concentrations corresponding to the expression of genes, PAs must play a vital role in ARD (see *5.5.*) as also in leaves PA biosynthesis genes revealed an upregulation in ARD variants starting from days 10-14. Other genes in leaves which were analyzed by RT-qPCR sometimes showed a time-dependent expression pattern but variants rarely revealed significant differences at one time point, hence genes that were found in the leaf MACE analysis (see **4.**) would present additional starting points to better understand ARD in regard of affected molecular reactions in the whole plant.

5.5. Potential enhanced role of PAs in ARD

Biotic factors play a predominant role in causing ARD and amongst them fungi were considered most important (Manici et al. 2013; Franke-Whittle et al. 2015). This was also highlighted by the high expression of PA biosynthesis genes under ARD conditions in this work. Gene expression was coupled with the extraordinary high amounts of biphenyls and dibenzofurans found in root material. Nevertheless, abiotic factors cannot be excluded totally and lately, more studies have been conducted in analyzing the role of allelochemicals and root exudates in ARD (Manici et al. 2016; Yin et al. 2016, 2017; Zhu et al. 2017).

Phenolic compounds belong to the rich group of secondary metabolites in plants and they are indicated by hydroxylated aromatic rings involved in disease resistance (Singh et al. 1999; Wu et al. 2001). They may be released by plants through leaching, root exudation, volatilization or decay of plant materials (Gur and Cohen 1989; Kuiters 1990; Wu et al. 2000; Politycka and Adamska 2003; Weir et al. 2004; Zhang et al. 2007; Baerson et al. 2008). They can enhance ROS production and damage the plant's antioxidant system under replant conditions (Yin et al. 2016; Zhu et al. 2017) ending with augmented membrane leakage in addition to disruption of amino acid and hormone metabolism (Weir et al. 2004; Gao et al. 2010) but on the other hand they can act as ROS scavengers as well (Henfrey et al. 2015).

It could be shown that phenols exuded by roots and decomposed roots of preceding cultures are one of the causes of ARD (Börner 1959; Bai et al. 2009; Gao et al. 2010; Nicola et al. 2016; Yin et al. 2016, 2017), pointing to phloretin, phloridzin and amygdalin inhibiting apple growth even in low concentrations and affecting microbial communities in soils (Börner 1959; Baerson et al. 2008; Jilani et al. 2008; Hofmann et al. 2009; Nicola et al. 2016; Yin et al. 2016, 2017). This may also be true for

the PAs found in this work as Yim et al. (2015) – working with the same soil – found bacterial degraders of phenolic compounds to be less abundant in ARD soils pointing to detrimental soil microbial community shifts. Fungi which are able to use the identified PAs as a recognition compound may therefore be activated or promoted as also suggested by other studies (Börner 1959; Hofmann et al. 2009; Cesco et al. 2012; Nicola et al. 2016). Phloridzin was found to promote growth of *Fusarium* spp. (Yin et al. 2017) which were found to produce allelochemicals negatively affecting plant growth possibly due to synergistic interaction of fusaric acid with other toxins suppressing development and biomass via reduced transpiration and photosynthesis rates (Bacon et al. 1996; Wu et al. 2008; Manici et al. 2016).

Moreover, phenols also react with the soil through ligand exchange reactions, oxidation and incorporation into organic matter in addition to soil sorption (Makino et al. 1996; Blum 1998; Blum et al. 1999; Jilani et al. 2008). Whereas the half-life of free phenols in soils is rather short, sorption to soil particles could lead to the build-up of increasing phytotoxic concentrations in the soil (Blum 1998; Blum et al. 1999). For PAs in particular it could be shown that biphenyl-degrading bacteria are environmentally omnipresent (Hernandez et al. 1995), and *Pseudomonas putida* as well as *Rhodococcus erythropolis* were able to process soil-sorbed biphenyls (Feng et al. 2000). This would hint at PAs having a potential long-term effect on plants by serving as a carbon source for microorganisms in ARD soils. Incidence of citrus replant disease could still be found after 10 years without growing citrus on site which led to the suggestion that allelopathic compounds involved in disease etiology could exist in a subsoil environment (Burger and Small 1983). Furthermore, it was concluded that due to a decline in beneficial fungi like *Trichoderma viride* which are capable of breaking down phenolic compounds, toxins would be able to accumulate resulting in plant growth reduction under sufficient concentrations (Burger and Small 1983). Hence, PAs may play a role as allelochemicals in the long-term effect on orchards via nurturing disadvantageous microbial community shifts.

Obviously, based on obtained results in this work it is not known if identified PAs are also exuded into the soil but in vitro experiments eliciting PAs from ‘M26’ have shown that PAs were excreted into the medium (cooperation Prof. Winkelmann and Prof. Beerhues, Hannover/Braunschweig, unpublished data). Hence, theoretically it is possible for ‘M26’ to exude PAs also into the soil and therefore leading to potential microbial community shifts.

Regardless, if the PAs were exuded into the soil, they were found in high concentrations in the roots of ARD variants. As discussed above (see **3.**), PAs may have led to a state of cytotoxicity due to possible missing detoxification systems. Likewise, related phenols like phtalic acid in *M. prunifolia* induced oxidative stress that resulted in cellular damage as well as growth inhibition (Bai et al. 2009).

Studies in *A. thaliana* have shown that the PA camalexin can be induced via PRR detection of MAMPs like peptidoglycan, flagellin and chitin plus ethylene, jasmonic and salicylic acid, MAPKs as well as ROS, often in interaction dependent pathways (Qutob et al 2006; Gust et al. 2007; Ahuja et al.

2012; Pandey et al. 2016). PTI might defend the plant from biotic stress in this regard but the cell wall based host defense might be tuned down by pathogens secreting effector proteins directly into the plant cells (Chizzali et al. 2012a). ETI could then be responsible for recognition of pathogens leading to defense reactions (Lee and Lu 2011). Chizzali et al. (2012a) concluded that cellular borders would have to be congested to block diffusion of toxins produced by infected cells or pathogens into neighboring cells (Lee and Lu 2011).

It was shown that ethylene can induce synthesis of PAs derived from the phenylpropanoid pathway (Kamo et al. 2000; Chung et al. 2001; Ishigaki et al. 2004). Further elicitation was observed for *Phytophthora* spp. which produced parasiticein and cryptogein in tobacco which activated a salicylate responsive kinase and other defense associated genes inducing PA production, stimulating an oxidative burst, proton influx and HR cell death (Zhang et al. 1998). Whereas, in potato, *Vicia fabia* and *Phaseolus vulgaris*, host resistance was correlated to the level of PA accumulation, *Brassica* spp. and various cruciferous plants lacked this relationship and high PA concentrations can potentially lead to cytotoxicity (Dixon et al. 1994; Rogers et al. 1996). Loss of tonoplast integrity leading to the release of toxic plant metabolites and hydrolytic enzymes was attributed to the PA phaseolin killing bean and beet cells (Glazener and Van Etten 1978; Hargreaves 1980) and also in *A. thaliana* the PA camalexin led to cytotoxicity (Rogers et al. 1996). As PAs were higher in ARD variants and for aucuparin and 3-hydroxy-5-methoxybiphenyl exclusively found in this variant, PTI must have worked for 'M26' plants. However, in a second step, it is probable that ETI did not work and biotic stressors were able to suppress the effect of PAs in stopping pathogens probably by the release of effectors or toxins as some fungi and oomycetes develop haustoria from which they can release such substances into the plant cell (De Coninck et al. 2014). Interestingly, resistance genes possibly responsible for the detection of such molecules were found in this study as well but they were not discussed in the submitted manuscripts. Twenty-one NBS-LRR genes with a FDR (false discovery rate) adjusted $p < 0.05$ were detected in roots (see **2.** Manuscript I, Supplementary Table S1). Although, only two of these genes showed at least a 2-fold downregulation in ARD samples (MDP0000185737 and MDP0000276078), in total 15 genes were of lower expression in ARD roots, while six genes were slightly upregulated. The higher amount of genes with lower expression in ARD variants supports the view that ETI might not work properly in ARD challenged 'M26' plants as genes for the potential detection of detrimental effectors were only expressed on a lower level in ARD roots.

Interestingly, ROS-triggered HRs leading to cell death or PA-induced cytotoxicity would favor the colonization of the root system by necrotrophic fungi (Pandey et al. 2016) which play an elevated role in causing ARD (Manici et al. 2013; Franke-Whittle et al. 2015). In addition, it was suggested that the recognition of MAMPs in roots would lead to a continuous activation of inducible defense responses which would cost the plant a lot of energy that could not be used for plant growth (De Coninck et al. 2014). The plant's fitness would be negatively affected as suggested by the proposed immunity-growth tradeoff which states that plants have to make a decision on how much

energy they are willing to sacrifice from growth related processes to defense against pathogens (Heil and Baldwin 2002; Huot et al. 2014).

Oxidation, hydroxylation, dealkylation and glutathione conjugation have been discussed in detoxification via glutathione S-transferases (GSTs), malonyltransferases, UDP-glucosyltransferases (UGTs) and cytochrome P450 dependent hydroxylases (CYPs). These general detoxification processes and involved enzymes were proposed to play a major role in the formation of non-toxic glycosides as well as glutathione and malonyl conjugates which can be deposited in vacuoles or associated with cell wall material (Kuiters 1990; Cole 1994; Singh et al. 1999; Inderjit and Duke 2003; Baerson et al. 2005; Gosch et al. 2010). H+-gradient-dependent transport and ATP-binding cassette (ABC) transporters are reported to be involved in the vacuolar sequestration of such glucosides (Yazaki 2005). Corresponding genes were not discussed in the submitted manuscripts, however, in total 10 GSTs, 15 UGTs and 21 CYPs with a FDR adjusted $p < 0.05$ were found in roots (see **2.** Manuscript I, Supplementary Table S1). Three GSTs were of lower expression in ARD whereas seven GSTs were upregulated in ARD samples but only one of these GSTs was upregulated more than 2-times (MDP0000161016; 2.59). Five UGTs were higher expressed in γARD samples, of which one showed a relevant fold change (MDP0000423529; -2.78), compared to 10 UGTs in ARD samples, of which one showed a fold change greater two (MDP0000160578; 2.59). Of the 21 CYPs, seven were downregulated in ARD samples – four were regulated more than 2-fold (MDP0000119148, MDP0000166337, MDP0000149604 and MDP0000215936) – whereas 14 showed higher expression in ARD samples, of which three revealed at least 2-times regulation (MDP0000184534, MDP0000879787 and MDP0000152900). In sum, this reveals a diverse expression behavior of genes with possible detoxification characteristics and it is fascinating to speculate about presumably impaired detoxification systems in 'M26' under ARD conditions but functional analysis of these genes is necessary to draw any definitive conclusions.

OMTs may play an important role in distributing PAs to the apoplastic space because alkylation of hydroxyl groups leads to changes in solubility impacting localization and biological activity of metabolites (Ibrahim et al. 1987; Zubieta et al. 2001; Berim et al. 2012). In this regard, glucosylation increases the water solubility of a compound, then accumulating in vacuoles whereas methylation decreases the water solubility and leads to the transport of a metabolite from the cell into the apoplastic space (Grotewold 2004; Yazaki 2005; Marinova et al. 2007). The sequestration or release into the apoplast via exocytosis is mediated by ABC membrane-associated transporters (Baerson et al. 2005). Chizzali et al. (2012a) speculated that BIS3 protein is associated with plasmodesmata due to its detection at the junctions between cortical parenchyma cells. The authors pointed to the importance of plasmodesmata in symplastic communication between neighboring cells as well as translocation of signaling molecules being involved in the coordination of growth and development and serving as potential gateways to spread infection from pathogens from cell to cell (Maule 2008; Lucas et al. 2009; Lee and Lu 2011). Seven ABC transporters with a FDR adjusted

$p < 0.05$ were found in roots (see **2.** Manuscript I, Supplementary Table S1) but none of them showed a regulation greater two, five were downregulated and two were upregulated in ARD samples.

Interestingly, so far, no glycosides of biphenyls and dibenzofurans were detected in intact plants of apple (Chizzali and Beerhues 2012; Chizzali et al. 2012b) and also in this study no glucosyl transformation of PAs could be detected on the metabolic level. Therefore, also probable compartmentation in vacuoles is unlikely and hints at the possibility of impaired detoxification systems in ARD susceptible 'M26' plants. To better understand the involvement of PAs in ARD, experiments should be performed in locating the expression of PA biosynthesis enzymes. When plants produce high amounts of PAs and are not able to detoxify the compounds, they probably kill themselves trying to defend against the biotic stressors.

Nevertheless, PAs cannot be regarded as the primary cause for ARD as disinfection of ARD soil resulted in improved apple plant growth. Biotic stressors in the rhizosphere are the main culprit for ARD. It now remains to be seen which microorganisms in particular trigger the PA biosynthesis in the ARD-susceptible apple rootstock 'M26'. Experiments testing the interaction of microorganisms with PA biosynthesis should be conducted. However, experimental designs will not be trivial as many potential causal agents have been reported in ARD and even the often reported genera of *Cylindrocarpon* spp., *Pythium* spp. and *Phytophthora* spp. include many different species. Nonetheless, Shin et al. (2014, 2016) infected apple seedlings with one of the potential ARD causal agents, namely *Pythium ultimum* and they were able to show differential regulation of several genes. These studies could serve as a blueprint in testing different reported ARD causal agents which could probably reduce the complexity of ARD inciting microorganisms majorly if differential regulation of PA biosynthesis genes could be observed.

5.6. Conclusion and outlook

Taking all the findings of this work into consideration together with results already reported in ARD, PAs seem to play an enhanced role in the disease etiology of the susceptible apple rootstock 'M26'. While leaves seemed to suffer from systemic oxidative stress mediated via biotic stress in roots, the accumulation of biphenyl and dibenzofuran PAs in the root system – for the first time reported in apple roots – may hint at impaired biotic stress responses in ARD challenged 'M26' plants. In this study it was clearly shown that biotic stressors affected the plant growth of 'M26' because γ-irradiation majorly improved the plants' habitus. Figure 5.1 presents an overview of the potential reactions involving PAs in ARD and resulting future research requirements. Most likely ARD causal agents led to the induction of PA biosynthesis genes (1) which requires the further elucidation of the specific ARD causal agents being responsible for the induction of PAs. The resulting products accumulated in the root system and reached concentrations that were extraordinary high compared to other studies (2). In addition, localized concentrations can be assumed to be even higher resulting in a possible state of cytotoxicity for plants as the detoxification of compounds was not possible either

because of insufficient/lacking systems or suppression via detrimental ARD microorganisms which has to be tested in future experiments. In addition, it remains to be seen if PAs may be excreted into the soil solution via exudation or leeching (3), and if potential sorption of PAs to soil particles is possible (4) presenting the microbiome with a possible long-term carbon supply for detrimental microbial community shifts (5) which also should be further investigated. Tolerant genotypes may possess the ability of efficiently using the well-established defense response of PA production, either through controlled induction or differing PA composition, plus they may inhabit potent detoxification systems which presents additional research questions to be followed as well.

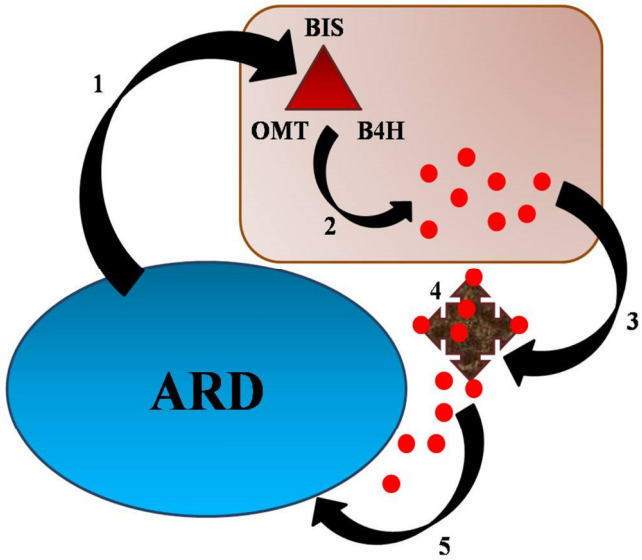


Figure 5.1 Hypothetical proposed reactions in ARD affected 'M26' roots and resulting future research requirements (1-5, see text). Microorganisms in ARD soil try to infect the root system which leads to the subsequent induction of PA biosynthesis genes encoding amongst others BIS, OMT and B4H enzymes (1). This results in the production of biphenyls and dibenzofuran PAs (red spheres) in high concentrations which could end in potential cytotoxicity (2). PAs might be exuded or leeched into the soil (3) where they could possibly bind to soil particles for long-term sorption (4) and might potentially be used by microorganisms being able to employ PAs as a carbon source leading to likely detrimental microbial community shifts further aggravating ARD (5).

The results obtained in this work demonstrate for the first time molecular reactions in ARD affected 'M26' plants. However, they can only serve as a base for further studies as the conclusions drawn from results are highly speculative at this point, even if they are fundamentally sound. First of all, future experiments have to test the hypothesis of potential PA cytotoxicity in 'M26' plants. This will be prevalent in determining the role of PAs in ARD. Furthermore, PAs should be studied in regard of potential detoxification, localization and exudation. Clearly, the obtained results have to be tested in other genotypes to better understand ARD in planta. Comparing more tolerant genotypes could eventually help in determining molecular markers for breeding and selection processes. Additionally, functional analysis of discussed genes may help in further understanding affected molecular reactions in ARD-challenged 'M26' plants. Finally, it should be mentioned that studies dealing with biotic stress should always link results obtained for plants with the biotic stressors themselves. Hence, inoculation experiments with certain ARD pathogens correlated to PA biosynthesis gene expression and metabolite accumulation as well as effects of PAs on microbial

characteristics could present an interesting option. As discussed in *5.5.*, it will also be interesting to see which microorganisms actually cause the high upregulation of PA biosynthesis genes.

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