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Transcriptional profiling of *Fraxinus excelsior* leaves during the early infection phase of ash dieback

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Abstract

Ash dieback (ADB) has been causing the progressive decline of Fraxinus excelsior trees throughout Europe, urging research and forest management to develop strategies to combat ADB. A genetically heritable component in susceptibility to this fungal disease was reported in common gardens. Thus, exploring the molecular basis of ADB susceptibility will further support breeding initiatives in the future. We performed transcriptional profiling of infected and uninfected leaves from two ash genotypes with different susceptibility to *Hymenoscyphus fraxineus*. Leaf rachises were sampled one week following inoculation. Differential gene expression analysis was performed to compare between treatments in each genotype (individual response) or in genotypes and treatments combined (common response). Due to the heterogeneity in the response, only DEGs were discussed that passed stringent assessment. Our results revealed that UW1, the most susceptible genotype, showed a total of 515 differentially expressed genes (DEGs), some of them possibly suggesting a self-control mechanism, hindering an effective immune response and causing increased susceptibility. On the other hand, FAR3, the least susceptible genotype with 230 DEGs, seemed to induce a contained but more efficient response, hinting toward a salicylic acid-mediated process and activating pathogen-related (like) proteins as thaumatin-like, peroxidases, and chitinases. In the common response, 512 DEGs were modulated and transcripts from the phenylpropanoid pathway were commonly altered in both genotypes. Altogether, this work comprised an initial transcriptional exploration including two selected genotypes with distinct susceptibility to ADB, however, the heterogenous response indicated the need to further improve the experimental inoculation approach. Exploring gene expression patterns in ADB susceptibility holds promise to reveal early response mechanisms, and new markers related to susceptibility, as well as to contribute to developing strategies that may help contain ADB.

Keywords Fraxinus excelsior · Hymenoscyphus fraxineus · Genotypes · Heritable susceptibility · RNA-seq

Introduction

Forest ecosystems shelter biodiversity and provide important ecosystem services such as the prevention of soil erosion, water regulation, and carbon storage (Thompson et al. 2011). However, higher demands for natural resources

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due to increases in human population, markets, worldwide trade, and transport in combination with climate change are strongly affecting forests. These factors have led to a loss of coverage and changes in the geographic distribution of the remaining forests (Ramsfield et al. 2016), and in this disrupted scenario, the transport and establishment of invasive

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insect and pathogen species has been facilitated (Simler-Williamson et al. 2019). Thus, several cases of severe tree diseases are currently known, such as the Dutch elm disease, sudden oak death, chestnut blight, white pine blister rust, and the dieback of European common ash (Budde et al. 2016).

Ash dieback (ADB), a lethal fungal disease, has been severely reducing ash populations over the last 30 years (Pautasso et al. 2013). Throughout Europe, common ash (Fraxinus excelsior L.) trees are important not only for their centuries-long cultural and economic value (Bell et al. 2008; Pratt 2017), but also due to their fundamental ecological role for many associated species (Enderle et al. 2019). In terms of distribution, ash is a strong colonizer, but rarely becomes a dominant species (Heuertz et al. 2004a). The population genetic structure of ash trees indicates a high gene pool diversity with restricted genetic exchange between populations in southeastern Europe and in Sweden, while a widespread gene pool is prevalent in central and western European populations (Heuertz et al. 2004b). In regions where a scattered distribution leads to limited gene flow between different locations, the species is considered potentially vulnerable to genetic erosion (Myking 2002; Heuertz et al. 2004a).

The first cases attributed to ADB were reported in the early 1990s in Poland, involving a high mortality of ash trees regardless of age (Kowalski 2006). Later, the ascomycete Hymenoscyphus fraxineus (previously named Chalara fraxinea and Hymenoscyphus pseudoalbidus for its asexual and sexual stages, respectively) was identified to be the causal agent (Baral et al. 2014). The disease cycle starts between June and September, when ascospores penetrate the leaf surface via appressoria, resulting in necrotic spots and, sometimes, leading to leaf shedding approximately 10-14 days later (Kräutler and Kirisits 2012; Cleary et al. 2013). In fall, black pseudosclerotia develop in petioles and leaf rachises in the litter from which white-stalked apothecia will develop during the following warm season and release new ascospores (Gross and Holdenrieder 2013; Timmermann et al. 2011). The infection leads to death in most ash trees and has spread over entire countries. In association with the decline of other tree species in flood plain forests, such as elms due to Dutch elm disease or alder and pedunculate oak due to various Phytophthora species (Pautasso et al. 2013), ADB now represents a major threat to European biodiversity.

Susceptibility to ADB is a polygenic and moderately heritable trait. Ash trees infected with *H. fraxineus* show genetically controlled responses, such as early flushing of the buds or early shedding of leaves in autumn, which have been correlated with ash dieback susceptibility (McKinney et al. 2011; Muñoz et al. 2016). Furthermore, when comparing damaged crowns of clones grown in different locations, a single genotype is usually affected to a similar degree regardless of the environment (McKinney et al. 2012). Overall, as reviewed in Enderle et al. (2019), several studies from progeny and clonal field trials reported narrow-sense (ranging from 0.37 to 0.53) and broad-sense heritability values (ranging from 0.10 to 0.65), corroborating a moderate genetic control of ADB susceptibility.

In this context, further research efforts to understand the mechanisms underlying susceptibility to ADB and to develop suitable conservation, forest management, and breeding programs are of utmost importance and have been considered a measure to deal with the ash dieback epidemic (Pautasso et al. 2013; Enderle et al. 2019). One key and cost-effective strategy to investigate biological systems is to study transcriptional modulations as a means to explore gene expression in response to biotic stressors (Wang et al. 2009). More specifically, this approach can lead to the identification of genes involved in the response to a pathogen attack, allowing the development of genetic markers for breeding programs (Müller et al. 2023). Still, the molecular basis of ADB susceptibility and its mechanisms have not been exhaustively studied, and the exploration of the transcriptional response to ADB is currently very limited, even though reference genomes of common ash are already available (Sollars et al. 2017; Meger et al. 2024). As examples, tissue colonization assessed by pathogen transcript monitoring following rachis inoculation in common (F. excelsior) and Manchurian ash trees (F. mandschurica Rupr.) have reinforced a correlation between field performance and the infection intensity, and also highlighted a role for the timing of metabolic response during the infection process (Nielsen et al. 2022). In a transcriptional profiling study of border regions of necrotic spots on ash bark 10 months after infection, it was possible to identify that the mevalonate and iridoid pathways are modulated following infection, also revealing modulated transcription factors (TFs) and genes that are part of the jasmonate signaling pathway (Sahraei et al. 2020). Moreover, a metabolite expression study on ash bark tissue revealed the two coumarins, fraxetin and esculetin, to be strongly associated with susceptibility (Nemesio-Gorriz et al. 2020).

To contribute to the future preservation and management of ash trees, the research network FraxForFuture was formed in Germany in 2020 (Langer et al. 2022). The current work is a part of the FraxGen subproject, and we focused on performing an initial RNA-Seq analysis of genotypes with different susceptibility to ash dieback, which were sampled during the early infection phase. Our work with mock-inoculated and pathogen-inoculated ash trees indicated that significant transcriptional changes could be detected already seven days after inoculation. Moreover, we compared lists of differentially expressed genes (DEGs) which were significantly modulated between control and infected groups for each genotype individually, also identifying commonly regulated DEGs in both genotypes. Overall, our aim was to start laying a foundation for transcriptomic studies on infected ash leaves under controlled conditions and, although we observed experimental variability, we reinforce the potential of transcriptionally investigating ash genotypes differing in susceptibility. This strategy will allow for the identification of genomic variants in candidate genes that could be used for marker-assisted selection and contribute to the development of new breeding strategies and conservation initiatives.

Material and methods

Plant material, growth conditions, experimental setup and data acquisition

The plant material was provided by the Bavarian Agency of Forest Genetics (Bayrisches Amt für Waldgenetik, AWG). European ash trees growing in a clonal garden in Grabenstätt have been monitored since 2014. At the time the adult trees were selected in the field (2010) they were all identified as less susceptible (Fig. 1). The two clones selected for this study (FAR3, UW1) were within the first third of less susceptible clones using long-term crown damage inspection. The FAR3 clone was identified as the least susceptible and the clone UW1 as susceptible, ranging in the middle compared to all other 35 clones within the trial (Seidel et al. in prep.; Fig. 1). The most susceptible and heavily affected genotypes could not provide a sufficient amount of scions for grafting, justifying the choice of UW1 instead. FAR3 was originally collected close to the town Haiming at approximately 48.21°N, 12.90°E, and UW1 was collected close to the town Unterwössen at approximately 47.72°N, 12.46°E.

Scions were cut in winter 2020/2021 and replicated by grafting in February 2021 onto 2-year-old *F. excelsior* trees as rootstocks. The ash trees used as rootstocks came from a nursery, provided by Erwin Vogt Baumschulen GmbH, Pinneberg, the provenance was north-west Germany, code 81,101. Plants were grown in 4 L plastic pots containing a commercially available substrate mixture "Profi-Linie mineralisch" from Kleeschulte Erden GmbH & Co. KG, Rüthen, with pH (CaCl₂) 6.0, Salinity 1.5 g/L, N 320 mg/L, P₂O₅ 120 mg/L, K₂O 350 mg/L, Mg: 120 mg/L. Six grafted trees per genotype were kept in a greenhouse with natural light conditions and semi-controlled humidity and temperature (Fig. 2A). Plants were repotted, fertilized, and watered regularly. The current experiment reported here was conducted in July 2021.



Fig. 1 European ash trees monitored since 2014 in a clonal common garden in Grabenstätt, in which the clone FAR3 was identified as the least susceptible and the clone UW1 as susceptible. **A** Percent of tree mortality for the two genotypes used in the present study. **B** Survival probability rate of ramets during the years of monitoring (Seidel et al. in prep.)

Pathogen inoculation and sampling

Isolates of *H. fraxineus* were tested for virulence and maintained in the culture collection at Julius Kuehn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Forest Protection, Braunschweig, Germany. The most virulent strain (Strain 7—RH03-T2-B1-1) was selected as the reference strain based on severe symptom development after stem and petiole inoculations in saplings (Ridley et al. 2024). The *H. fraxineus* reference strain culture has been deposited in the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 116307.

The reference strain was cultivated at room temperature for approximately three weeks on MYP medium (prepared by mixing 2.8 g malt, 0.4 g peptone, 0.2 g yeast, 6 g agar, 400 ml ultrapure water, and circa 5 g ash leaves). Autoclaved wooden toothpicks (approx. 0.5 cm) were cultivated together with the pathogen for two weeks and used for inoculation (Fig. 2B, C). Toothpicks were placed inside a 1 cm-long superficial wound in the rachis (for a



Fig. 2 Schematic of the experimental inoculation procedure. **A** Greenhouse with potted *Fraxinus excelsior* trees, which were grafted from scions originating from individuals with different susceptibility to ash dieback disease. **B** Culture plates of *Hymenoscyphus fraxineus*

morphological reference of the leaf regions, see Nielsen et al. 2022) (Fig. 2D) and sealed with parafilm (Fig. 2E). Autoclaved toothpicks incubated on sterile medium were used as controls. Collection of leaf samples took place after seven days following the inoculation, although there were no clear signs of necrosis development yet. The rachis region comprising the wound, in addition to 1 cm distally and proximally, respectively, from the inoculation wound were flash-frozen in liquid N₂ and kept at -60 °C until further use. Three biological replicates for each genotype were sampled.

RNA isolation and sequencing

For each sample 40–60 mg of rachis tissue were ground without thawing using a Retsch MM300 (F. Kurt Retsch, Haan, Germany). RNA was extracted using the E.Z.N.A. Plant RNA Kit Omega Bio Tek (R6827-01, Norcross Georgia, USA) according to the manufacturer's protocol for difficult samples. The concentration and purity of the RNA were assessed using a microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, United States), and values between 1.8 and 2.2 for $A_{260/280}$ nm and

strain 7—RH03-T2-B1-1. C Wooden stick covered with *H. fraxineus*. D A 1 cm superficial wound on the rachis of ash leaves was cut with a scalpel. E A wooden stick was placed on the wound. F Sealing of the wound by wrapping the wooden stick with parafilm

 $A_{260/230}$ ratios were considered sufficiently pure. In case these ratios were slightly lower than the optimal, a precipitation protocol was performed, involving overnight incubation at -20 °C with absolute ethanol, followed by centrifugation at 4 °C, two consecutive washes with 70% ethanol and centrifugation at room temperature, and resuspension with nuclease-free water.

Total RNA samples were treated with DNase I (Thermo Scientific) and the RNA quality was assessed using a Fragment Analyzer System (PROSize 3.0, 3.0.1.5, 2015, Advanced Analytical Technologies, Agilent Technologies). cDNA libraries were prepared with the Stranded mRNA Prep kit (Illumina, San Diego, USA), which uses oligo(dT) magnetic beads for purifying and capturing polyA tails from mRNA molecules. Libraries were sequenced using the Illumina HiSeq4000 platform, 50 bp and single-end reads.

Bioinformatic processing and differential gene expression analysis

The bioinformatic analyses were carried out in a High-Performance Computing (HPC) Center from the Gesellschaft für Wissenschaftliche Datenverarbeitung mbH Göttingen (GWDG), which is based on GNU/Linux and scheduled by Simple Linux Utility for Resource Management (SLURM; Yoo et al. 2003).

FastQC (v0.11.7), MultiQC (v.1.10.1) (Ewels et al. 2016), and Trimmomatic (v0.36) (Bolger et al. 2014) were used for quality control, removing adapter sequences, and trimming of 12 nucleotides from the head and 2 nucleotides from the tail of all reads. Reads shorter than 20 nucleotides and presenting an average quality score below 15 within a sliding window of 4 bases were filtered out. Subsequently, we used Hisat2 v.2.1.0 (Kim et al. 2019) to map fastq files to the F. excelsior reference genome version FRAX_001_PL (downloaded from https://www.ncbi.nlm.nih.gov/datasets/ genome/ GCA_019097785.1/, Meger et al. 2024). Mapping was performed with the option –score-min L,0,-0.2, and mapped files were processed using Samtools v.1.9 (Li et al. 2009). Finally, counting was performed with HTSeq (v.2.0.2; Anders et al. 2015), using the GFF3 file obtained from the FRAX_001_PL version of the genome to indicate positions of mRNA in the genome (Meger et al. 2024). The final count-table was imported to R (R Core Team 2022) for downstream analysis.

The R package DESeq2 (Love et al. 2014) was employed to perform differential expression analysis. DESeq2 uses a Wald test to assess significance of negative-binomially distributed RNA-Seq data, with *P* values adjusted by the false discovery rate (FDR; Benjamini and Hochberg 1995). It also uses logarithmic fold change (LFC) values, which were defined as the logarithm of the ratio between treated (infected in this case) and untreated (control) samples. Expression values were first normalized using the variance stabilizing transformation (vst) method implemented in DESeq2, and transformed data were examined using a principal component analysis (PCA). Two approaches were used for expression analysis:

- The "individual response" for each genotype was assessed comparing infected and control plants. Those transcripts with an adjusted *P* value below the standard 0.05 and showing a log₂ fold change (LFC) >|1| were considered as Differentially Expressed Genes (DEGs). Volcano plots of the individual responses were generated with the R package EnhancedVolcano v.1.14 (Blighe et al. 2018). The two sets of DEGs obtained for each genotype were compared using a Venn diagram plotted with the R package ggVennDiagram v.1.2.2 (Gao et al. 2021).
- The "common response" was analyzed by including the genotype factor as another variable in the DESeq2 formula design (see section Availability of Data and Code). DEGs were considered significant for adjusted *P* value < 0.05 and LFC > 111 for at least one of the geno-

types. The results were visualized in a heatmap of the z-scores (calculated as the ratio between the mean and the standard deviation of each DEG) generated with the R package pheatmap v.1.0.12 (Kolde 2019).

We selected and discussed in particular genes with LFC >|2| and adjusted *P* value < 0.01 that were involved in immune responses, phenylpropanoid biosynthesis, mevalonate pathway, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) signaling pathways, as well as transcription factors (TFs) (Zamora-Ballesteros et al. 2021; Islam et al. 2022).

Functional annotation of DEGs, enrichment analysis, and gene selection

For the functional annotation of DEGs, we created a local protein database from the RefSeq Viridiplantae section at the National Center of Biotechnology Information (NCBI). A total of 41,355 gene model sequences from the genome version FRAX_001_PL were used (Meger et al. 2024) and included in a multi-fasta file used as input for BLASTp (Camacho et al. 2008). A maximum number of 50 hits per sequence was set, with an *e* value threshold of $10e^{-5}$, and results were imported into Omicsbox v3.0.30 (Götz et al. 2008) to complete the annotation with the Functional Analysis module. Annotation with GO terms encompassed the Mapping, Annotation, and GO-Slim steps, in addition to InterProScan which also retrieves GOs associated with protein motifs and domains. Functional annotation of the 41,355 gene models developed for the reference ash genome (FRAX 001 PL) resulted in 41,032 annotated transcripts (99.22%), while only 323 sequences (0.78%) could not be annotated for either Blast Hits, GO Mapping, GO Annotation or GO-Slim (Table 1). The complete annotation is shown in Supplementary Table 2.

Enriched GOs were identified by a two-tailed simple enrichment analysis (SEA) performed for all DEGs (the two individual responses for FAR3 and UW1, and the common response). The significance assessment was carried

 Table 1
 Overview of annotated sequences for gene models included in *Fraxinus excelsior* reference genome FRAX_001_PL

Annotation steps	Number of sequences
Total	41,355
Not annotated	323
Only with Blast Hits	495
Only with GO Mapping	7997
Only with GO Annotation	6552
With GO-Slim	25988

out, following Fisher's exact test featured by Omicxbox (Al-Shahrour et al. 2004), and using as reference the complete annotation in GO terms for all gene models.

Results

Ash genotypes could be clearly distinguished via transcriptome sequencing

An average of 32 million reads were generated for each sample (Supplementary Table 1). After trimming of lowquality transcripts and removal of contaminants, an average of approx. 31 million reads per sample were kept and from those, an average of 89.83% were mapped against the reference genome assembly FRAX_001_PL of *F. excelsior*. Total transcripts after quality filtering were used in a principal component analysis (Fig. 3). The first two principal components (PCs 1 and 2) explained 46% and 31% of the variance observed, respectively, and the genotypes UW1 and FAR3 could be easily distinguished along PC1. Biological replicates for the control group also showed a high conformance in PC2, whereas replicates for the pathogen-inoculated treatments showed a scattered distribution.

DEGs revealed distinct patterns of modulation between genotypes

After processing and annotation of sequenced reads, we performed separate differential gene expression analyses: the first to individually compare the modulations occurring



Fig. 3 Principal component analysis (PCA) of transcripts for the two genotypes of European ash (*Fraxinus excelsior*) showing higher (UW1) and lower (FAR3) susceptibility to ash dieback disease. Rachises were inoculated with the pathogen using wood plugs and allowed to grow for a week, when sampling of control (solid circles) and infected (solid triangles) groups occurred. Different colors indicate different genotypes and treatments

in genotypes UW1 (higher susceptibility) and FAR3 (lower susceptibility), and second, the two genotypes jointly. This two-step approach sought to monitor DEGs in response to infection either in each individual genotype, or commonly modulated in both genotypes (see Methods section).

Figure 4a, b shows a graphical representation of the significant DEGs (adjusted *P* value < 0.05; LFC >|11) in a comparison between control and treatment for each genotype. Patterns differ considerably, with a wider distribution of DEGs for UW1 (Fig. 4b). While a total of 230 DEGs were significantly modulated in FAR3, 515 were found in UW1. Among those, 136 were up- and 94 down-regulated in FAR3, whereas 444 were up- and 71 were down-regulated in UW1. The complete lists of identified DEGs for both genotypes and their respective LFC values and adjusted *P* values are provided in Supplementary Tables 3 and 4.

We compared the DEGs identified in each genotype and only nine genes were common between FAR3 and UW1, representing 1.2% of the total DEG number (Fig. 4c, Supplementary Table 5). These common genes included three WRKY transcription factors (TFs) (gene12571, gene14628, gene23853), an 1B-like ethylene-responsive TF (gene46078), a glucan endo-1,3-beta-glucosidase (gene41383), an alpha carbonic anhydrase (gene47080), a chloroplastic polyphenol oxidase I (gene51554), a 47 kDa synapse-associated protein (gene56171), and a suppressor of disruption of TFIIS (gene52496).

From the lists of DEGs for each genotype (Supplementary Tables 3 and 4), we selected and discussed in particular genes with LFC>|2| that were involved in immune responses, such as phenylpropanoid biosynthesis, mevalonate pathway, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) signaling pathways, and transcription factors (TFs) (Table 2). Most significant DEGs were up-regulated, but UW1 showed more down-regulated DEGs (34) than FAR3 (21). In the immune response category, FAR3 showed only thaumatin-like proteins and basic endochitinase-like, whereas UW1 also had several E3 ubiquitin-protein ligase proteins and a disease resistance response protein 206-like. In terms of secondary metabolites, although a similar number of DEGs was found for both genotypes in the category biosynthesis of phenylpropanoids, UW1 exclusively showed a 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1-like from the mevalonate pathway, as well as a putative 12-oxophytodienoate reductase 11 as part of JA pathway. Numerous transcripts with varied functions in ET pathway were also up-regulated in UW1 (Table 2). Lastly, all five identified TFs in FAR3 were WRKYs, while for UW1 a diverse repertoire of TFs showed LFC > -2 (Table 2).

Separating the DEGs in up- and down-regulated sets per genotype did not yield any significantly enriched GO terms (results not shown). Therefore, we used the two full sets of DEGs (from FAR3 and UW1;





Fig. 4 Differential gene expression of two European ash genotypes that are more (UW1) or less (FAR3) susceptible to ash dieback. Leaf rachises of trees were inoculated with sterile or infected wood plugs and sampled after seven days. **A, B** Volcano plots for FAR3 (A) and UW1 (B). X-axis indicates \log_2 -fold change values; y-axis indicates the $-\log_{10}$ of *P* values. Red circles indicate genes with adjusted *P* values of <0.05 and LFC>III. **C** Venn diagram of differentially expressed genes (DEGs) which are up- or down-regulated with adjusted *P* values of <0.05 and LFC>III. Blue color scale indicates the number of DEGs. **D** Enriched GO terms revealed after simple enrichment analysis (SEA) using the list of DEGs for UW1. Overrepresented GOs are those with a higher percentage of sequences in

Supplementary tables 3 and 4) to perform a two-tailed SEA of GO terms. While no significantly enriched GO terms were identified for FAR3 (Supplementary Table 6), 11 enriched terms were found for UW1 (Fig. 4d; Supplementary Table 7). From these 11 GOs, six from the Cellular Component (CC) category were under-represented (GO:0005622, "intracellular anatomical structure "; GO:0005634 "nucleus"; GO:0043226, "organelle"; GO:0043227, "membrane-bounded organelle";

the test (list of DEGs) than in the reference (complete list of gene models), while the percentage of sequences in under-represented GOs is higher in the Reference set. The description for the highlighted GO terms is as follows: GO:0019748 secondary metabolic process; GO:0005622 intracellular anatomical structure; GO:0005634 nucleus; GO:0043226 organelle; GO:0043227 membrane-bounded organelle; GO:0043229 intracellular organelle; GO:0043231 intracellular membrane-bounded organelle; GO:0003723 RNA binding; GO:0003824 catalytic activity; GO:00164910xidoreductase activity; GO:0016740 transferase activity. The SEA results for FAR3 were not significant and are therefore not shown. *BP* biological process; *CC* cellular component; *MF* molecular function; *GO* Gene Ontology

GO:0043229, "intracellular organelle"; GO:0043231, "intracellular membrane-bounded organelle"), together with one GO from the Molecular Function (MF) category (GO:0003723, "RNA binding"). On the other hand, over-represented GOs included three terms from the MF category (GO:0003824, "catalytic activity"; GO:0016491, "oxidoreductase activity"; GO:0016740, "transferase activity") and one from the Biological Process (BP) category (GO:0019748, "secondary metabolic process"). **Table 2** Selected DEGs (LFC>|2|) from the individual response analysis and classified per category. The list is classified based on increasingLFC per category. Negative LFC means down-regulation and positive LFC, up-regulation

Gene ID	Description	LFC	adjusted P value
Immune respons	e		
FAR3			
gene41134	peroxidase 42-like [Olea europaea var. sylvestris]	2.07	0.043890762
gene10295	thaumatin-like protein [Olea europaea var. sylvestris]	2.10	0.005621391
gene35979	basic endochitinase-like [Olea europaea var. sylvestris]	2.60	0.028011333
gene16958	peroxidase 12-like [Olea europaea var. sylvestris]	3.08	0.001018325
gene55223	thaumatin-like protein [Olea europaea var. sylvestris]	3.25	0.007501784
gene35829	basic endochitinase-like [Olea europaea var. sylvestris]	3.56	0.006722346
UW1			
gene19486	probable E3 ubiquitin-protein ligase RHA4A [Olea europaea var. sylvestris]	-2.16	0.000528699
gene42370	probable LRR receptor-like serine/threonine-protein kinase At1g53440 [Olea europaea var. sylvestris]	2.27	0.040923708
gene45192	E3 ubiquitin-protein ligase WAV3-like isoform X1 [Salvia splendens]	2.53	0.000516016
gene24831	E3 ubiquitin-protein ligase PUB23-like [Olea europaea var. sylvestris]	2.60	0.000226903
gene39449	E3 ubiquitin-protein ligase RHA2A-like [Olea europaea var. sylvestris]	2.64	9.68284E-06
gene13983	E3 ubiquitin-protein ligase PUB23-like [Olea europaea var. sylvestris]	2.81	1.19797E-05
gene4656	thaumatin-like protein 1b [Olea europaea var. sylvestris]	3.00	0.001886435
gene21955	disease resistance response protein 206-like [Olea europaea var. sylvestris]	3.13	0.001271404
gene50396	thaumatin-like protein [Olea europaea var. sylvestris]	4.47	0.00049578
gene40032	E3 ubiquitin-protein ligase RHA2B-like [Olea europaea var. sylvestris]	4.80	0.010060147
gene4044	E3 ubiquitin-protein ligase RHA2B-like [Olea europaea var. sylvestris]	6.10	0.001713104
- Biosynthesis of p	henylpropanoids		
FAR3			
gene27831	alpha-glucosidase 2-like isoform X1 [Salvia hispanica]	-2.25	0.038617805
gene51842	glucan endo-1,3-beta-glucosidase 11-like [Olea europaea var. sylvestris]	2.02	0.031951988
gene49788	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	2.31	0.003413624
gene11936	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	2.40	8.06383E-09
gene57748	cationic peroxidase 1-like isoform X1 [Olea europaea var. sylvestris]	2.44	1.28637E-05
gene56752	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	2.89	3.59041E-09
gene49784	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	3.01	0.027452279
gene41383	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	4.09	8.06383E-09
gene44168	beta-glucosidase-like isoform X2 [Olea europaea var. svlvestris]	5.18	0.006463636
UW1			
gene20843	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	-2.69	0.023729714
gene16388	anthocyanidin 3-O-glucoside 2 ^{'''} -O-xylosyltransferase-like [Olea europaea var. sylvestris]	2.04	0.019145355
gene43130	4-coumarate–CoA ligase 2-like [Olea europaea var. sylvestris]	2.28	0.003825206
gene46772	phenylalanine ammonia-lyase-like isoform X1 [Olea europaea var. sylvestris]	2.39	0.025302066
gene55647	phenylalanine ammonia-lyase-like isoform X1 [Olea europaea var. sylvestris]	2.41	0.032549987
gene49783	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	2.66	0.034830271
gene41383	glucan endo-1.3-beta-glucosidase, acidic-like [Olea europaea var, sylvestris]	3.15	0.001423023
gene1044	beta-glucosidase-like isoform X2 [Olea europaea var. sylvestris]	3.79	0.01085737
gene16778	lignin-forming anionic peroxidase-like [Olea europaea var. sylvestris]	6.86	0.006838628
MVA nathway			
UW1			
gene13507	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1-like [Olea europaea var, sylvestris]	4.18	0.000503877
Transcription fac	etors (TFs)		
FAR3	()		
gene12571	probable WRKY transcription factor 50 [Olea europaea var sylvestris]	2.63	0.014620399
vene?4961	probable WRKY transcription factor 43 [Olea europaea var sylvestris]	3.46	0.017570707
0-1102 1901	processe	5.40	5.01/5/0/0/

Table 2 (continued)

Gene ID LFC Description adjusted P value 0.044879943 gene46078 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 3.55 gene23853 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 4.60 0.000561876 gene14628 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 4.84 0.015247718 UW1 gene44089 zinc finger protein CONSTANS-LIKE 1-like [Olea europaea var. sylvestris] -3.140.004062066 gene53663 transcription factor HBI1-like isoform X1 [Olea europaea var. sylvestris] -2.800.004181453 gene43402 zinc transporter 1-like [Olea europaea var. sylvestris] -2.533.78953E-08 ethylene-responsive transcription factor RAP2-3-like [Olea europaea var. sylvestris] 2.01 gene6760 0.000363731 zinc finger protein ZAT11-like [Olea europaea var. sylvestris] 0.047851691 gene8650 2.16 gene41945 zinc finger protein ZAT10-like [Olea europaea var. sylvestris] 2.20 0.013287144 gene52243 transcription factor bHLH149-like [Olea europaea var. sylvestris] 2.29 0.001423023 transcription factor MYB108-like [Olea europaea var. sylvestris] gene33741 2.31 0.00073802 gene8649 zinc finger protein ZAT12-like [Olea europaea var. sylvestris] 2.32 0.046620757 gene27665 zinc finger CCCH domain-containing protein 15-like [Coffea arabica] 0.006228774 2.50 gene24497 transcription factor MYB87-like [Olea europaea var. sylvestris] 2.54 0.021966165 transcription factor bHLH78-like isoform X1 [Olea europaea var. sylvestris] 0.02753492 gene7310 2.59 gene53219 homeobox-leucine zipper protein ATHB-52-like [Andrographis paniculata] 2.83 0.032532312 gene17325 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 2.01471E-05 2.87 gene27964 ethylene-responsive transcription factor 1A-like [Olea europaea var. sylvestris] 3.00 0.043138797 gene15014 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 3.01 0.033389049 gene12571 probable WRKY transcription factor 50 [Olea europaea var. sylvestris] 3.01 0.008329368 gene21136 NAC transcription factor 32-like [Olea europaea var. sylvestris] 3.17 1.70503E-10 transcription factor MYB108-like [Olea europaea var. sylvestris] 0.006654049 gene21335 3.24 transcription factor MYB108-like [Olea europaea var. sylvestris] 0.008020111 gene19400 3.30 ethylene-responsive transcription factor ABR1-like [Olea europaea var. sylvestris] gene45787 3.32 0.014179135 gene39851 transcription factor MYB114-like [Olea europaea var. sylvestris] 3.40 0.006499166 0.007025816 gene813 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 3.41 gene45311 NAC domain-containing protein 79-like [Olea europaea var. sylvestris] 9.0762E-05 3.54 gene44635 probable WRKY transcription factor 40 [Olea europaea var. sylvestris] 3.78 7.65368E-10 gene51097 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 3.79 0.00177755 homeobox-leucine zipper protein HOX11-like isoform X2 [Olea europaea var. sylvestris] gene23287 3.81 0.020894037 gene1711 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 4.06 0.004062066 gene46078 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 4.34 0.047109291 transcription factor bHLH93-like [Olea europaea var. sylvestris] gene6009 4.36 1.96561E-05 gene56031 zinc finger protein GIS3-like [Olea europaea var. sylvestris] 4.39 0.000224679 gene2954 ethylene-responsive transcription factor 1B-like [Olea europaea var. sylvestris] 4.46 0.00985783 gene25015 ethylene-responsive transcription factor ABR1-like [Olea europaea var. sylvestris] 4.90 0.000194924 gene25842 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 2.19796E-05 4.98 gene48533 NAC domain-containing protein 2-like [Olea europaea var. sylvestris] 3.36893E-06 5.28 gene27363 transcription factor bHLH162-like [Olea europaea var. sylvestris] 0.026761909 5.34 gene23853 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 5.54 5.08046E-06 5.96 gene56601 transcription factor MYB8-like [Olea europaea var. sylvestris] 0.021710579 gene25119 ethylene-responsive transcription factor ERF110-like [Olea europaea var. sylvestris] 6.06 0.016848626 gene14628 probable WRKY transcription factor 75 [Olea europaea var. sylvestris] 6.40 0.001125969 Jasmonic acid pathway UW1 gene37552 putative 12-oxophytodienoate reductase 11 [Sesamum indicum] 2.54 0.008228671 Salicylic acid pathway FAR3 gene33656 salicylic acid-binding protein 2-like [Olea europaea var. sylvestris] 2.01 0.014346101

Table 2 (continued)

Gene ID	Description	LFC	adjusted P value
gene6437	protein DMR6-LIKE OXYGENASE 2-like [Olea europaea var. sylvestris]	4.27	0.020499253
UW1			
gene33633	salicylic acid-binding protein 2-like [Olea europaea var. sylvestris]	2.63	0.001371967
Ethylene pathway			
UW1			
gene43829	1-aminocyclopropane-1-carboxylate oxidase-like [Olea europaea var. sylvestris]	2.80	0.00011535
gene53843	1-aminocyclopropane-1-carboxylate synthase-like [Olea europaea var. sylvestris]	3.26	2.63325E-05
gene12137	1-aminocyclopropane-1-carboxylate synthase-like [Olea europaea var. sylvestris]	3.54	1.24589E-05
gene43841	1-aminocyclopropane-1-carboxylate oxidase-like [Olea europaea var. sylvestris]	3.57	3.42964E-14
gene28206	1-aminocyclopropane-1-carboxylate synthase-like [Olea europaea var. sylvestris]	4.06	4.60667E-05
gene11437	1-aminocyclopropane-1-carboxylate oxidase 1-like [Olea europaea var. sylvestris]	4.29	0.000104275
gene2877	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like [Olea europaea var. sylvestris]	4.30	5.92876E-09

DEGs commonly regulated by both genotypes

After considering the individual modulations that took place in each genotype, we then repeated the differential gene expression analysis including both genotype and treatment as variables (see Methods). A total of 512 significant DEGs were identified and are listed in Supplementary Table 8. To further explore this list, we performed a hierarchical clustering using z-scores transformation per transcript, and four main clusters could be identified (Fig. 5a). Although there was a slight difference in the level of the response between biological replicates within clusters, common patterns of up- or down-regulation were sufficiently conserved between the treatments. There were a higher number of strongly upregulated genes in the infected groups, as seen in clusters 1 and 3 for both genotypes (Fig. 5a).

We were then interested in comparing the three lists of DEGs generated from the common versus the individual response approaches. The nine genes presented in the individual analysis were also identified in the common analysis (Fig. 5b). 220 DEGs corresponding to 23% of the total were exclusively identified in the common response analysis, while 69 for FAR3 and 214 for UW1 were also found in the individual response approach (Fig. 5b; Supplementary Table 9). In total, as shown in the Venn diagram, 152 DEGs (15.9%) for FAR3 and 292 (30.5%) for UW1 were only identified in the individual analysis.

Next, we screened the resulting common response list for strongly modulated DEGs (LFC >|2|) using the same keyword-set used to categorize DEGs in the individual response (Supplementary Table 8). Among the DEGs that differed from the individual analysis, eight leucine-rich repeat (LRR) proteins were found (gene1307, gene13877, gene15810, gene27011, gene30540, gene41689, gene44119, and gene49570), either up- or down-regulated in both genotypes (Table 3). Additionally, gene6437 and gene6456 were annotated as protein DMR6-LIKE OXYGENASE 2-like and were strongly up-regulated in FAR3 and UW1, and several components of the phenylpropanoid pathway were significantly modulated (Table 3).

Lastly, we performed a two-tailed SEA of DEGs from the common response (Supplementary Table 8) resulting in eight enriched GO terms (Fig. 5c; Supplementary Table 10). Over-represented terms belonged to the BP (GO:0009719, "response to endogenous stimulus"; GO:0042221, "response to chemical"; GO:0050896, "response to stimulus") and MF (GO:0003824, "catalytic activity") categories, while terms from the CC category were found to be under-represented (GO:0043226, "organelle"; GO:0043227, "membranebounded organelle"; GO:0043229, "intracellular organelle"; GO:0043231, "intracellular membrane-bounded organelle").

Discussion

Among research efforts to halt ash dieback spread, understanding the genetic diversity of the host underlying heritable susceptibility is of key importance for future breeding and management initiatives (Pautasso et al. 2013). In the present study, we selected two ash genotypes with lower (FAR3) and higher (UW1) susceptibility to ADB originated from natural German ash populations. Our study showed that the more susceptible genotype UW1 showed more than twice as many DEGs (515) in the individual response as the less susceptible FAR3 (230). Among UW1 DEGs, several transcripts involved in cell wall metabolism and lignification were modulated (e.g., laccase-1-like, laccase-15-like; laccase-14-like; cinnamoyl-CoA reductase 1-like; cellulose synthase-like protein E6, among others, Supplementary Table 4), including the overrepresentation of the GO term secondary metabolic process. In conifers, lignification increases during fungal infection to prevent hyphal



Fig. 5 Analysis of the common response between European ash genotypes and comparison to each genotype individually. Leaf rachises from ash trees with higher (UW1) and lower (FAR3) susceptibility to ash dieback were sampled after seven days of inoculation with infected and uninfected wooden plugs. The analysis for the common responses used both genotypes and treatments as variables for the differential expression analysis, whereas the individual response compared treatments for each genotype separately. A Hierarchical clustering of differentially expressed genes (DEGs). Color scale from blue to orange indicates z-scores of transformed (vst) values per transcript. Groupings for genotype, treatment and cluster numbers are shown. **B** Venn diagram of DEGs comparing the analysis for the common and individual responses. Values show DEGs up- or down-regulated at adjusted P value < 0.05 and log₂ fold change (FC)>III. Color scale indicates the number of DEGs. The

penetration, especially in *Pinus* species resistant to *Fusarium* circinatum, and a role for phenylalanine ammonia-lyase (PAL) in linking primary and secondary metabolism has been reported (Zamora-Ballesteros et al. 2021). Here, however, we observed an opposite pattern, with lignification-related transcripts being most prominent in the susceptible ash genotype. This could also be a response to the trophic behavior of the pathogen, as *F. circinatum* is a necrotrophic species (Morse et al. 2004) and forces the host to actively lignify the infected area, while *H. fraxineus* follows an initially biotrophic strategy (Mansfield et al. 2019). Since *H. fraxineus* presents both a necrotrophic and biotrophic phase of infection, clearly there is a complex nature of the host genotype-dependent response in *F. excelsior* (Gross et al. 2014).

In addition, our analysis revealed significantly upregulated transcripts for the 3-hydroxy-3-methylglutarylcoenzyme A reductase 1-like (LFC = 4.18, adjusted P

same nine DEGs shown in Fig. 3 are also at the intersection here. **C** Enriched GO terms identified using simple enrichment analysis (SEA) using the list of DEGs for the common response. Over-represented GOs are those with a higher percentage of sequences in the test (list of DEGs) than in the reference (complete list of gene models), while the percentage of sequences in under-represented GOs is higher in the reference set. The description for the highlighted GO terms is as follows: GO:0009719 response to endogenous stimulus; GO:0043221 response to chemical; GO:0050896 response to stimulus; GO:0043226 organelle; GO:0043227 membrane-bounded organelle; GO:0043231 intracellular membrane-bounded organelle; GO:003824 catalytic activity *BP* biological process; *CC* cellular component; *MF* molecular function; *GO* Gene Ontology

value 0.0005) in UW1 (Table 2). This transcript encodes an enzyme that catalyzes the synthesis of mevalonate in the isoprenoid pathway in plants (Learned and Fink 1989). As an important marker, it has been reported that the production of iridoid glycosides, which are terpenoid-derivatives, was lower in less susceptible ash trees (Sambles et al. 2017; Sollars et al. 2017). Iridoid glycosides are involved in antiherbivory functions and, hence, were reported to be present in higher concentrations in *F. excelsior* leaves under the attack of emerald ash borer (Chen et al. 2011; Qazi et al. 2018). This may mean, however, that breeding for lower iridoid glycosides may increase the susceptibility to other biotic agents (Sambles et al. 2017).

In terms of secondary metabolism, phenylpropanoids are an essential part of the defense metabolism (Zaynab et al. 2018). Here, we observed that both genotypes showed up-regulation of several DEGs involved in phenylpropanoid biosynthesis, differing in the fact that FAR3, the less
 Table 3
 Selected DEGs from the common response analysis and classified per category. Only results that differed from the individual response analysis are shown. Negative LFC values indicates down

regulation and positive LFC, up-regulation. The complete list is available in Supplementary Table 7

Gene ID	Description	adjusted P value	LFC, FAR3	LFC, UW1		
Immune response						
gene1307	leucine-rich repeat extensin-like protein 6 [Olea europaea var. sylvestris]	0.0020062	-1.62	-1.95		
gene13877	probable LRR receptor-like serine/threonine-protein kinase At1g56140 isoform X1 [Olea europaea var. sylvestris]	0.0170021	-1.50	-1.42		
gene15810	probable LRR receptor-like serine/threonine-protein kinase At5g63710 isoform X1 [Olea europaea var. sylvestris]	0.029179	-1.35	-1.11		
gene27011	probable LRR receptor-like serine/threonine-protein kinase At5g63710 isoform X2 [Olea europaea var. sylvestris]	0.0270392	1.00	1.41		
gene30540	LRR receptor-like serine/threonine-protein kinase EFR [Olea europaea var. sylvestris]	0.0479516	1.21	0.99		
gene41689	leucine-rich repeat extensin-like protein 3 [Olea europaea var. sylvestris]	0.049509	1.67	3.17		
gene44119	probable LRR receptor-like serine/threonine-protein kinase At2g23950 [Olea europaea var. sylvestris]	0.0301822	1.83	0.59		
gene49570	LRR receptor-like serine/threonine-protein kinase [Olea europaea var. sylvestris]	0.0234073	2.35	3.06		
gene4656	thaumatin-like protein 1b [Olea europaea var. sylvestris]	0.0314467	2.08	3.20		
gene55223	thaumatin-like protein [Olea europaea var. sylvestris]	0.0363116	3.06	0.73		
Salicylic ac	id pathway					
gene6437	protein DMR6-LIKE OXYGENASE 2-like [Olea europaea var. sylvestris]	0.0173918	3.90	3.70		
gene6456	protein DMR6-LIKE OXYGENASE 2-like [Olea europaea var. sylvestris]	0.0301204	3.93	3.45		
Biosynthesis of phenylpropanoids						
gene43130	4-coumarate-CoA ligase 2-like [Olea europaea var. sylvestris]	0.0161129	1.78	1.74		
gene1044	beta-glucosidase-like isoform X2 [Olea europaea var. sylvestris]	0.0343985	-2.38	-2.44		
gene11936	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	0.0156	-1.71	-1.75		
gene41383	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	0.0226535	1.64	4.27		
gene49782	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	0.0436593	2.42	4.30		
gene49783	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	0.0253309	2.42	3.06		
gene49787	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	0.0453388	2.42	2.37		
gene49788	caffeic acid 3-O-methyltransferase-like isoform X1 [Olea europaea var. sylvestris]	0.0051741	2.42	0.81		
gene56752	glucan endo-1,3-beta-glucosidase, acidic-like [Olea europaea var. sylvestris]	1.9E-06	3.40	4.38		

susceptible genotype, presented up-regulation of a peroxidase 42-like and down-regulation of an alpha-glucosidase 2-like isoform X1, whereas UW1 showed down-regulation of caffeic acid 3-O-methyltransferase-like isoform X1. Several genes involved in the phenylpropanoid pathway were also significantly differentially expressed in the common response analysis, further confirming the modulation in both genotypes. A similar response including specific peroxidase modulations, has been reported in both resistant and susceptible genotypes of American elm infected with *Ophiostoma novo-ulmi* (Islam et al. 2022). Thus, phenylpropanoid metabolism alone does not appear to be able to distinguish between trees with different susceptibility to ADB.

Plants have evolved a complex immune system based on two levels. The first is the pattern-triggered immunity (PTI) and is activated upon recognition of pathogen elicitors, such as pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) by transmembrane pattern recognition receptors (PRRs). However, pathogens have also developed molecular mechanisms to block PTI by using effectors, inducing the so-called effector-triggered susceptibility (ETS). Therefore, the second level of plant immunity involves the expression of R (resistance) genes after ETS, encoding proteins such as nucleotide-binding leucine-rich repeats (NB-LRRs) (Jones and Dangl 2006). NB-LRRs recognize pathogen effectors and induce the effector-triggered immunity (ETI), which in turn activates downstream cascades and enhances PTI (Yuan et al. 2021). In the common response analysis performed here, several LRR proteins were up- and down-regulated (Table 3), suggesting the activation of ETI and subsequent potentiation of PTI in both genotypes. The PTI+ETI response activates phytohormone biosynthesis. Responses mediated by JA and ET are more common in cases of necrotrophic pathogens, whereas the SA-dependent pathway is mostly activated in biotrophic infections (Glazebrook 2005; Huang et al. 2020). It has also been shown that there is a temporal balance with blurred boundaries between JA, ET, and SA-mediated responses,

especially when attacked by hemibiotrophic fungi (Ding et al. 2011).

FAR3, the less susceptible genotype, showed concomitant up-regulation of salicylic acid-binding protein 2-like (LFC = 2.01) and protein DMR6-like oxygenase 2-like (LFC = 4.27). DMR6-like oxygenase has been reported to suppress the immune response by repressing the SA pathway, which has been previously connected to susceptibility to mildew infections (Zeilmaker et al. 2015). Although DMR6-like oxygenase was also up-regulated in the common response analysis, we considered it more relevant in the context of FAR3 alone, since other hormone-related pathways were not induced at similar levels in this genotype. This may indicate a more stringent control in FAR3 of the hypersensitive response (HR), which is triggered by SA, and the subsequent programmed cell death (PCD) in the pathogeninfected area, promoting necrosis and limiting further spread of the infection by nutrient deprivation, but also negatively affecting host tissues (Balint-Kurti 2019). Indeed, the higher peroxidase activity and expression of reductases in FAR3 might also be related to detoxification processes and scavenging of reactive oxygen species (ROS), involved in the initiation of the HR. Finally, the most up-regulated TFs in FAR3 belonged to the WRKY family, which are reported to be involved in immune responses and to mediate SA and JA responses (Glazebrook 2005; Eulgem and Somssich 2007). In particular, WRKY75 regulates JA responses against necrotrophic pathogens (Chen et al. 2021). Although different homologs of WRKY75 were present in both genotypes, higher LFC values were found in the less susceptible genotype FAR3. Overall, it seems that a JA-mediated response may control the switch from biotrophic to necrotrophic behavior of *H. fraxineus*, while a SA-mediated response, inducing the production of PR-proteins and subsequently phenylpropanoids, is also activated in the early stages of ADB. A similar response has been observed in the case of Dutch elm disease, which affects the European field elm (Ulmus minor Mill.) (Chano et al. in prep.).

In our study, FAR3 showed six up-regulated transcripts (LFC>2) classified as part of the immune system, annotated as thaumatin-like proteins (gene55223 and gene55223), peroxidases-like proteins (gene41134 and gene16958), and basic endochitinase-like proteins (gene35979 and gene35829) (Table 2), which classify as pathogen-related (PR) proteins. Thaumatin-like proteins and peroxidases are assigned to PR-5 and PR-9 and show reportedly antifungal roles (Jain and Khurana 2018). Chitinases are actually PR-like proteins (PRLs), usually involved in fungal cell wall degradation, and have been induced by SA-mediated responses in pine seedlings challenged by the necrotrophic *Fusarium subglutinans* f. sp. *pini* (Davis et al. 2002).

In contrast to this concise and restrained response, several DEGs annotated as E3 ubiquitin-protein ligases (i.e., types

RHA4A, RHA2B, WAV-3, PUB23) were up-regulated in UW1, leading to the overrepresentation of the GO terms catalytic and transferase activity. Ubiquitination is a posttranslational modification that feedbacks and controls plant immune responses, since, when in excess, immune responses may be detrimental to plant growth and promote autoimmunity by unbalanced HR and PCD (You et al. 2016; Chen et al. 2022). Interestingly, GO terms for endomembrane trafficking were under-represented in UW1 (Supplementary Figure S1), supported by up-regulation (LFC = 2.6) of the E3 ubiquitin-protein ligase PUB23-like, which is reported to attenuate chitin-elicited responses by controlling endomembrane trafficking of plant immune receptors, thereby reducing PAMP-triggered responses (Stegmann et al. 2012; Chen et al. 2022). Transcripts for PR-5 (thaumatin-like proteins) and a disease resistance response protein 206-like were up-regulated in UW1, the latter also reported to support the resistance of melon (Cucumis melo) to Fusarium wilt disease (Yang et al. 2022). Among the TFs strongly upregulated in UW1, the bHLH family has been reported to be involved in iridoid metabolism in Catharanthus roseus (Van Moerkercke et al. 2015). As well, up-regulated transcripts for numerous ET-responsive TFs and 1-aminocyclopropane-1-carboxylate oxidase-like/synthase-like suggested an ETmediated immune response, which would be expected for necrotrophic pathogens such as H. fraxineus. Therefore, one possible interpretation is that, in the more susceptible genotype UW1, the elicited response is fast and strong, triggering a self-controlled mechanism that may ultimately limit the effectiveness of the defense against the infection, and contribute to increased susceptibility.

The current work focused on the transcriptional changes in F. excelsior trees that occur in the leaf rachis 7 days after ADB infection, representing, to the best of our knowledge, one of the earliest samplings for RNA-seq performed in ash after infection. We used the inoculation procedure described by Schwanda and Kirisits (2016), but they evaluated the first symptoms after 20 days. When using a transcriptomic approach to study molecular host responses to pathogen infections, it is critical to focus on the initial steps of the disease (Arce-Leal et al. 2020). Although molecular changes can occur within seconds, here we considered seven days as an early stage of the infection since the disease develops and shows symptoms over the course of months in nature (Gross and Holdenrieder 2013). In the present study, seven days allowed for the detection of significant transcriptional changes when comparing control and infected groups of samples. At the same time, no GO enriched terms were found for one of the genotypes, and these results combined may reinforce that transcriptional changes associated with ash dieback in ash trees take place slowly, as also suggested for tanoak (Hayden et al. 2014), further indicating that seven days post-infection is probably the minimum period to start monitoring expressional changes in response to ADB. Mansfield et al. (2018) performed ascospore inoculations on F. *excelsior* leaves and reported the first visible symptoms already after seven days.

Although different pathosystems are very speciesdependent, similar monitoring times have been chosen in other tree species, such as in tanoak infected with *Phytophthora ramorum* (Hayden et al. 2014) and *Pinus pinaster* under *Fusarium circinatum* challenge (Hernandez-Escribano et al. 2020). Samplings during shorter incubation times have also been performed, as in samples taken only 96 h after inoculation with *Ophiostoma novo-ulmi* on American elms (Islam et al. 2022), and after only one and two hours post-inoculation with *Erwinia amylovora* on apple (*Malus* x *domestica*) (Norelli et al. 2009). These studies, like the present one, indicate it is possible to evaluate the early stages of the infection with useful outcomes.

Furthermore, we observed that the general trends in transcriptional modulation were consistent within each genotype (Fig. 5a), with some individual variation between our replicates (Fig. 3). This could be explained by temporal differences in the success of the infection process, reinforced by the absence of symptoms in some of the replicates (data not shown), resulting in a heterogeneous time frame of response between individual trees. Another source of variation may be a small but different amount of pathogen material, which may have grown differently on the wooden sticks used for inoculation. We highlight this as a potential limitation of working with wooden sticks as inoculants. In addition, we worked with grafted replicates of the same genotype with individual rootstocks. Ideally, plants derived from in vitro culture or rooted cuttings should be used to avoid rootstock effects.

Finally, the monitoring of the exact time point after infection is a challenging feature in natural populations, highlighting the value of studies under controlled conditions to characterize the molecular mechanisms of host and pathogen responses in a timely manner. However, set-ups have differed in the literature in terms of type of inoculation, tissue, and duration of the experiments, leaving room for multiple strategies that may influence the results. Here, we artificially inoculated leaf rachises with wood plugs and found a detectable transcriptional response seven days later, even though signs of necrosis were not visible at that time. In previous attempts to assess ADB susceptibility, ash wood plugs were used for bark inoculations at breast height and the disease spread was monitored for two years (Lobo et al. 2015). In another experiment testing for a phylogenetic signal in disease susceptibility, leaf and stem inoculations with ash wood plugs were monitored for 6-18 months in European, Asian and North American ash species (Nielsen et al. 2017). Moreover, by selecting uninfected ash branches and performing bark inoculations with infested agar plugs, a transcriptional profiling of the tissue was

performed after 10 months (Sahraei et al. 2020). In another study, ash petioles were sampled following rachis inoculation with ash wood plugs after three months to compare variance in disease susceptibility using quantitative polymerase chain reaction (qPCR) assays (Nielsen et al. 2022). In fact, the typical and natural way of infection for ADB is through ascospores landing on the leaf surface, but no transcriptional study has focused on this aspect so far. In particular, a focus on the typical pathway of infection over the leaf surface might reveal insights into early response mechanisms. However, ascospore solutions as inoculants are challenging, because ascospores are only viable for a short time window, which may reduce inoculation efficacy (Schlegel et al. 2016; Mansfield et al. 2018).

In summary, the aim of the current study was to provide initial insights into the early transcriptional component of susceptibility to ADB. It is clear that future experiments including a higher number of genotypes with distinct levels of susceptibility and sampling at different time points after inoculation will contribute to a wider and deeper understanding of the molecular mechanisms in response to ADB. Following more robust experiments to confirm and possibly select new DEGs involved in heritable susceptibility to ADB using transcriptomics, further verification in independent experiments using additional genotypes and methods to target specifically these DEGs, such as qPCR will be necessary.

Conclusions

We investigated the transcriptional changes in two ash genotypes from Germany with different susceptibility levels. Our work with mock-inoculated and pathogen-inoculated ash trees indicated that significant changes at the transcriptional level could be detected seven days after the start of the experiment. This work allowed for a comparison of analytical approaches, in terms of differential expression analysis, including both variables (genotypes + treatments), or control versus infected groups for each genotype. Overall, transcriptomics provided a valid approach to hint toward a stronger but possibly less targeted metabolic response in the more susceptible genotype (UW1), which may be more JA- and ET-mediated but may have caused a feedback loop that hindered the plant immune response. In contrast, the less susceptible genotype appeared to respond in a more targeted manner, mediated exclusively by the SA pathway, which might be more efficient and thus contribute to reduced susceptibility.

Availability of data and code

The sequencing data from the twelve samples used in this work have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession no. PRJNA1024896, SRA accession no. from SRR26302913 to SRR26302924 (http://www.ncbi.nlm.nih.gov/sra/). The code and scripts used for this article can be found at https://github.com/vchano/fraxgen_assay.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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