



Differential gene expression of *Hymenoscyphus fraxineus* grown on *Fraxinus excelsior* and *F. mandshurica* supplemented media

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Abstract

Ash dieback is caused by the invasive fungal species *Hymenoscyphus fraxineus* and leads to the loss of the common ash (*Fraxinus excelsior*) in many areas. In contrast, the pathogen seems to colonize the local ash species *Fraxinus mandshurica* (Manchurian ash) symptom-free in its native environment East Asia. In order to gain insight into the differences in the substrate dependence of the pathogen, two *H. fraxineus* strains (1511 and 1431) were grown in media supplemented with leaf material of *F. excelsior* or *F. mandshurica* and without supplement. A total of 95 and 916 genes were upregulated in media containing *F. excelsior* leaf material for strain 1431, compared to *F. mandshurica* and media without plant supplementation. The differential expression analysis of strain 1511 revealed 483 and 875 upregulated genes. Oxidative phosphorylation is highly upregulated in strain 1431 cultivated in *F. excelsior*-supplemented media indicating an active metabolism but probably also linked to the release of reactive oxygen species. In both strains during growth on *F. excelsior*, transcripts with similarity to genes of toxin biosynthetic pathways of other Ascomycota are enriched. In strain 1511, a gene cluster with similarity to depudecin cluster is upregulated in addition. For both strains, the upregulated genes, if grown in media supplemented with leaf material from *F. excelsior*, include a gene of the biosynthetic cluster of phytotoxin viridiol. The results suggest increased metabolic activity and toxin production in the presence of *F. excelsior* tissue compared to *F. mandshurica*.

Keywords Ash dieback · Substrate-dependent gene expression · Transcriptome · Toxin production

Introduction

Ash dieback disease is threatening the common ash (*Fraxinus excelsior*) in central Europe. The invasive ascomycete *Hymenoscyphus fraxineus* is causing this disease leading to decreased growth, reduced wood quality and declining ash trees. Transmission occurs by wind-borne ascospores, first colonizing the leaves and at a later stage invading the petioles and shoots (Timmermann et al. 2011; Cleary et al. 2013; Fones et al. 2016; Haňáčková et al. 2017) but also via root system (Fones et al. 2016; Baxter et al. 2023). Subsequent expansion in tissue can lead to necrosis of cambium and bark, shoot death or stem foot necrosis (Kowalski and Holdenrieder 2008; Schumacher et al. 2010; Peters et al. 2023). The fungi remain in leaves, and in autumn it lives as a saprotroph degrading foliage and forming new spores for

the infection cycle in the new year (Fones et al. 2016). In contrast, in its native environment East Asia the pathogen seems to colonize the local ash species *Fraxinus mandshurica* without any symptoms (Zhao et al. 2012). In Europe, the Manchurian ash shows only mild symptoms (Drenkhan and Hanso 2010; Nielsen et al. 2017).

It remains unclear why some ash tree species and even different genotypes of the same species as shown for *F. excelsior*, react more susceptible and some more resistant to *H. fraxineus* (Stener 2013; Cleary et al. 2014). Therefore, previous studies analyzed the transcriptional response of *F. excelsior* trees infected with *H. fraxineus*. Initial evidence has been obtained from transcriptome analyses that molecular markers can be used to identify individual trees with a low level of susceptibility to ash dieback (Harper et al. 2016; Sollars et al. 2017). Also, numerous single nucleotide polymorphisms (SNPs) have been identified in the ash genome that are associated with tree susceptibility (Stocks et al., 2019). Another study focusing on transcriptional responses of *F. excelsior* trees to *H. fraxineus* infection showed limited differences in gene expression patterns between resistant and

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susceptible *F. excelsior* clones (Sahraei et al. 2020). However, a set of candidate genes likely involved in the containment of the necrosis had been identified. The mechanisms of resistance are not yet well understood.

On the other hand, also little is known on the pathogen side. To gain more insights into the pathogen's genetic repertoire and pathogenic mechanisms, the genome and transcriptome of *H. fraxineus* has been compared with its close relative *H. albidus* which is native in Europe and non-pathogenic (Stenlid et al., 2017). It was shown that the two genomes are very similar and differences in gene expression may be responsible for the difference in interaction with the host. Especially, a pectin-degrading enzyme was identified in the *H. fraxineus* compared to *H. albidus* transcriptome that could be responsible for the degradation of cell walls and thus lead to a stronger host response. In addition, two small, secreted proteins were identified that were not found in *H. albidus*, one of which, cerato-platanin, could act as pathogen-associated molecular patterns (PAMPs).

In the genome of *H. fraxineus* a viridiol biosynthetic gene cluster had been identified (Elfstrand et al. 2021). Despite that viridiol showed necrotic activity on ash seedlings (Andersson et al. 2010; Cleary et al. 2014), its role in virulence is not clarified as it is also encoded and produced by non-pathogenic *H. albidus* (Junker et al. 2014; Elfstrand et al. 2021). Furthermore, the concentration of viridiol was also shown not to correlate with virulence (Junker et al. 2014). Another compound shown to be encoded only by *H. fraxineus* is hymenosectin (Elfstrand et al. 2021). It belongs to 3-decalinoyltetramic acid family, known to have broad spectrum antimicrobial and cytotoxic effects (Halecker et al. 2014). As it was isolated from crude extracts of virulent *H. fraxineus* strains, it is suggested as possible pathogenicity factor which could also be involved in defending competing fungi and bacteria.

As summarized above, several studies on transcriptional response of *F. excelsior* trees infected with *H. fraxineus* had been performed, and also *H. fraxineus* genome and gene expression in *F. excelsior* were analyzed and compared to *H. albidus*. Nevertheless, the underlying molecular processes in the pathogen–host interaction are not yet understood in detail. But it is supposed that the fungus expresses different genes—depending on the ash species. It was also shown that the growth rates of *H. fraxineus* on culture media supplemented with leaf tissue are largely consistent with the susceptibility of ash species to the pathogen (Carrari et al. 2015; Harper et al. 2016). However, the transcriptome of *H. fraxineus* strains during growth on different ash species has not been studied so far. This research is needed to gain insights into substrate and species-specific regulated genes, which contribute significantly to the differences in disease progression.

This study investigates the differential gene expression via RNA-Seq of *H. fraxineus* grown on media without and with leave supplements of *F. excelsior* and *F. mandshurica*. The aim was to identify species and substrate-specific regulated genes including virulence factors of the pathogen, and their implications on colonization, invasion and for the exploitation of nutrients.

We hypothesize that susceptible *F. excelsior* provides better growth conditions to *H. fraxineus* due to beneficial plant metabolite composition and that better growth conditions lead to a higher expression of genes for pathogenicity. To verify or disprove the hypothesis, changes in gene expression profile of *H. fraxineus* grown on media supplemented with plant material of susceptible (*F. excelsior*), more resistant (*F. mandshurica*) ash species and a control media were investigated.

Material and methods

Plant material and fungal culture

H. fraxineus isolates 1431 (2013–3-B1-21) and 1511 (2013–3-B15-6), provided from Northwest German Forest Research Institute (NW-FVA), Göttingen, Germany, were cultured on malt-yeast-peptone (MYP) agar. For RNA-Seq culture a liquid MYP media was supplemented with 36 g/l leave material of *F. excelsior* (EX) and *F. mandshurica* (MA) and MYP media without leave supplements was used as control media. Leave material of *F. excelsior* and *F. mandshurica* had been collected in August 2020 (isolate 1511) and 2021 (isolate 1431) from trees in the botanical garden at the University of Hohenheim, Stuttgart.

H. fraxineus isolates 1431 and 1511 were incubated at 20 °C for 28 and 24 days, respectively. 10 ml culture of isolate 1431 and 2 ml of isolate 1511 vortexed liquid culture had been centrifuged (10,000 × g for 10 min), and pellets were stored at – 80 °C.

RNA templates

The pelleted fungal mycelia were homogenized in Fast-Prep-24 5G (MP Biomedicals, Eschwege, Deutschland) with 8 m/sec, and total RNA was extracted by solid phase extraction of RNA using Invitrap Spin Universal RNA Mini Kit (Invitex, Berlin, Germany). The purified pellet was dissolved in 50 µl of double-distilled water and stored at –80 °C. The concentration of the total RNA was measured using Qubit® fluorometer with RNA HR Assay Kit (Invitrogen, Carlsbad, Germany) according to manufacturer's instructions. In addition, integrity of RNA had been assessed in agarose gel (1%), stained with GelRed (Genaxxon bioscience, Ulm, Germany), and visualized with a UV transilluminator.

RNA sequencing and automated downstream analysis

Three replicates were selected of each EX, MA, and MYP media cultures of *H. fraxineus* isolate 1511 and isolate 1431. After quality control, *H. fraxineus* isolate 1511 replicates were pooled, and 1431 replicates were used as triplicate for transcriptome studies. RNA sequencing and bioinformatic analysis was conducted by Novogene (UK) Company Limited, Cambridge, UK. Construction of mRNA libraries was performed by poly(A) enrichment. Illumina sequencing was carried out on NovaSeq 6000 with paired-end reads and 150 nt length (6 G raw data/ sample). The raw reads had been filtered to remove (a) reads containing adapters, (b) reads containing over 10% not determined bases (N > 10%) or (c) reads with a q score of over 50% bases ≤ 5 . The quality-passed reads were used for the subsequent analyses. The mapping on reference genome *H. fraxineus* nf4 (GenBank assembly accession: GCA_911649665.1) (Elfstrand et al. 2021) was done with HISAT2 (Kim et al. 2019). Total mapping rate has been calculated as follows: (mapped reads)/(total reads)*100. The assembly was done with StringTie (Pertea et al. 2015) and quantification of gene expression with featureCounts. DESeq2 (Love et al. 2014) was used for differential gene expression analysis with biological replicates and edgeR (Robinson et al. 2009) if no biological replicates had been included. For both methods a screening threshold of $|\log_2(\text{FoldChange})| \geq 1$ and adjusted p-value (padj) ≤ 0.05 had been applied to all genes in the compare groups.

The original gene annotation (Elfstrand et al. 2021) (<https://zenodo.org/record/4355824>) had been supplemented by UniProtKB Swiss-Prot database analysis (The Uniprot Consortium 2023) and Pfam protein families and domains (Mistry et al. 2021).

Identification of genes involved in toxin production and analysis of upregulated genes of interest

Protein sequences of the known biosynthetic gene clusters for gliotoxin in *Aspergillus fumigatus* (AY838877.1), hypothemycin in *Hypomyces subiculosus* (EU520418.1), aflatoxin in *Aspergillus parasiticus* (AY371490.1), and depudecin in *Alternaria brassicicola* (FJ977165.1) were used to identify putative homologue proteins potentially involved in toxin production in *H. fraxineus*. BLASTp algorithm was used to identify sequences showing a high structure similarity indicated by high e-values. For all *H. fraxineus* genes of interest, a new protein BLAST search of UniProtKB Swiss-Prot database was performed (June 2023). Additionally, Pfam protein families and domains had been verified with InterProScan (Jones et al. 2014). The protein sequences used

for all analyses were obtained from Elfstrand et al. 2021 (<https://zenodo.org/record/4355824>).

Putative effector genes

Differentially expressed genes (DEGs) had been analyzed in silico with Phobius (Käll et al. 2007) for transmembrane topology and signal peptides to identify putative effectors.

The comparative transcriptome analysis allows the identification of substrate-dependent differentially expressed genes of *H. fraxineus* to gain insights into the different courses of infection and identified key genes.

Results

Sequencing and mapping

RNA sequencing resulted in 89,081,550 total reads for isolate 1511 (pooled samples) grown on malt-yeast-peptone media (MYP) as control. For media supplemented with plant material from *F. excelsior* (EX) 93,701,440 and *F. mandshurica* (MA) 87,261,106 total reads had been obtained. Isolate 1431, which had been analyzed in triplicate, resulted in minimum of 39,856,930 total reads for MYP, 36,384,148 for EX and 40,040,864 for MA (Table 1). The percentage of total clean reads assigned to the *H. fraxineus* genome is higher than 92% for all samples.

Differential gene expression (DEG) analysis

DEG analysis was conducted to detect *H. fraxineus* transcriptome changes between growth on media supplemented with leaves from susceptible and resistant ash species to identify host specifically expressed genes. The comparison to control media allows to assess the tropism and influence of leave material.

For strain 1431, during growth on *F. excelsior* supplemented media compared to control media, a total of 1,777 genes had been differentially expressed, including 916 upregulated and 861 downregulated genes. Compared to *F. mandshurica* supplemented media, there are 126 DEGs, of which 95 genes are upregulated and 31 downregulated. Between growth on *F. mandshurica* and control media, there are 1,783 DEGs, with 841 up- and 942 downregulated genes.

For strain 1511, there are 1,837 DEGs between growth on *F. excelsior* supplemented media and control media, of which 875 are upregulated and 962 downregulated. There are 1,273 DEGs between *F. excelsior* and *F. mandshurica* supplemented media, with 483 upregulated and 790 downregulated genes. Comparing transcripts from growth in *F. mandshurica* supplemented media and control media, results in 812 DEGs, of which 424 are upregulated and 388

Table 1 Summary of the total illumina sequence reads (clean) and amount of data obtained from *Hymenoscyphus fraxineus* isolate 1511 and 1431 grown on malt-yeast-peptone media (MYP) as control andMYP media supplemented with plant material from *Fraxinus excelsior* and *Fraxinus mandshurica*

Media & Supplement	<i>H. fraxineus</i> 1511 (Pooled samples)			<i>H. fraxineus</i> 1431 (Triplicate)		
	Total clean reads	Amount of data	Total assigned reads (%)	Total clean reads	Amount of data	Total assigned reads (%)
Malt-yeast-peptone media	89,081,550	13.36 G		42,345,016	6.35 G	92.42
			94.11	44,554,196	6.68 G	93.92
				39,856,930	5.98 G	93.71
MYP supplemented with plant material from <i>Fraxinus excelsior</i>	93,701,440	14.06 G	94.89	36,384,148	5.46 G	94.91
				41,017,940	6.15 G	94.65
				41,068,466	6.16 G	94.87
MYP supplemented with plant material from <i>Fraxinus mandshurica</i>	87,261,106	13.09 G	94.43	48,137,624	7.22 G	94.68
				40,040,864	6.01 G	94.72
				48,042,912	7.21 G	94.63

downregulated. The number of predicted DEGs between different *H. fraxineus* comparison groups is shown in Table 2. To test the hypotheses in this study, only the upregulated genes of *F. excelsior* comparison groups are considered.

Upregulated genes of *H. fraxineus* grown on *F. excelsior* supplemented media compared to *F. mandshurica* supplemented media and MYP control media

We hypothesized that *H. fraxineus* colonization on susceptible *F. excelsior* may be associated with better growth due to beneficial plant metabolite composition. This will promote pathogenicity and will be reflected by changes in gene expression profile compared to colonization on resistant *F. mandshurica*. Therefore, differential gene expression of *H. fraxineus* had been compared between growth on *F. excelsior* supplemented media (EX) and control media (MYP) to assess tropism and influence of leave material. The comparison between the both supplemented media (EX and MA) provided the host-specific DEGs. The comparison was made

for both strains 1431 and 1511 (Table 3; Fig. 1). Additionally, the shared upregulated genes between EX vs. MYP and EX vs. MA had been assessed for both strains.

A total of 916 genes in *H. fraxineus* 1431 were found to be significantly upregulated in *F. excelsior* media compared to MYP control media (mean log₂FoldChange of 1.76, ± SD 0.83) and 95 genes in *F. excelsior* media compared to *F. mandshurica* media (mean log₂FoldChange of 2.52, ± SD 1.13). The comparison of *F. excelsior* media to MYP control media and *F. mandshurica* media resulted in 54 intersecting genes (Online Resource 1), whereof 22 genes (40.74%) are annotated as proteins of unknown function.

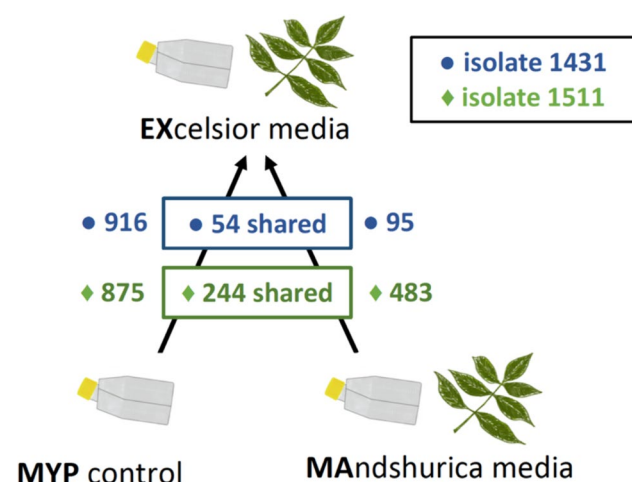
For strain 1511, there are 875 genes upregulated in EX compared to MYP (mean log₂FoldChange of 2.04; ± SD 1.20) and 483 genes in EX compared to MA (mean log₂FoldChange of 2.31; ± SD 1.62). There are 244 intersecting genes between both in EX upregulated gene sets (Online Resource 2). Among them are many genes (136 genes, 55.74%) which are encoding for proteins of unknown function.

Table 2 Number of predicted DEGs from *H. fraxineus* isolate 1511 and 1431 grown on maltose-yeast-peptone media (MYP) and MYP media supplemented with plant material from *Fraxinus excelsior* (EX) and *Fraxinus mandshurica* (MA)

Comparison groups	DEGs			Threshold
	All	Up	Down	
1431				
EX vs. MYP	1,777	916	861	DESeq2 padj <= 0.05 log2FoldChange >= 1.0
MA vs. MYP	1,783	841	942	DESeq2 padj <= 0.05 log2FoldChange >= 1.0
EX vs. MA	126	95	31	DESeq2 padj <= 0.05 log2FoldChange >= 1.0
1511				
EX vs. MYP	1,837	875	962	edgeR padj <= 0.05 log2FoldChange >= 1.0
MA vs. MYP	812	424	388	edgeR padj <= 0.05 log2FoldChange >= 1.0
EX vs. MA	1,273	483	790	edgeR padj <= 0.05 log2FoldChange >= 1.0

Table 3 Upregulated genes of *Hymenoscyphus fraxineus* isolate 1511 and 1431 grown in maltose-yeast-peptone media (MYP) supplemented with plant material from *Fraxinus excelsior* (EX) compared to MYP

<i>H. fraxineus</i> isolate	Comparison	Number of upregulated genes	Mean log2FoldChange values ($\pm$ SD) of upregulated genes	Shared upregulated genes
1431	EX vs. MYP	916	1.8 ($\pm$ 0.8)	54
	EX vs. MA	95	2.5 ($\pm$ 1.1)	
1511	EX vs. MYP	875	2.0 ($\pm$ 1.2)	244
	EX vs. MA	483	2.3 ($\pm$ 1.6)	

**Fig. 1** Upregulated genes of *Hymenoscyphus fraxineus* isolates 1511 and 1431 grown in maltose-yeast-peptone media (MYP) supplemented with plant material from *Fraxinus excelsior* (EX) compared to MYP media and MYP media supplemented with plant material from *Fraxinus mandshurica* (MA)

Analysis of those two shared upregulated gene datasets ($n = 54$ and $n = 244$) identified no commonly upregulated genes between both strains. Probably the limited number of predicted upregulated genes ($n = 95$) for comparison group *F. excelsior* (EX) vs. *F. mandshurica* (MA) and thus the small set of shared genes between treatments of strain 1431 prevent the identification of shared genes between strains.

Oxidative phosphorylation

Among the upregulated genes in strain 1431, there are many predicted to belonging to mitochondrial oxidative phosphorylation enzymes, like NADH-ubiquinone oxidoreductase (complex I), ubiquinol-cytochrome c oxidoreductase or cytochrome bc1 complex (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V). All the upregulated genes seem to be in a gene cluster encoded by mitochondrial DNA.

Many of the upregulated genes are predicted as subunits of the NADH-ubiquinone oxidoreductase respiratory

media and MYP media supplemented with plant material from *Fraxinus mandshurica* (MA). The mean of log2FoldChange and standard deviation ($\pm$ SD) of upregulated genes is indicated

complex like chain 1 (HYFRA_G00013582), chain 2 (HYFRA_G00013648), chain 4 (HYFRA_G00013619), chain 4L (HYFRA_G00013634), and chain 5 (HYFRA_G00013627). This complex I is the largest of the respiratory chain and the major entry point for electrons. Of complex III, the only protein located on mitochondrial DNA, cytochrome b (*cob*, HYFRA_G00013579) is predicted as upregulated. The genes *cox1*, *cox2*, and *cox3* are the largest subunits of cytochrome c oxidase (complex IV) and the ones which are mitochondrial encoded. In the upregulated gene set, there is a predicted subunit 1 (HYFRA_G00013653) and two subunits 2, one containing a periplasmic domain (Pfam: PF00116, HYFRA_G00013638), and one containing a transmembrane domain (Pfam: PF02790, HYFRA_G00013639). Furthermore, two predicted subunits of ATP synthase (complex V) of FO part, ATP6/ subunit a (HYFRA_G00013644) and ATP9/ subunit c (HYFRA_G00013622, HYFRA_G00013581), which are encoded by the mitochondrial genome are upregulated.

In addition, in the same gene cluster as the mitochondrial oxidative phosphorylation enzymes, there are four upregulated genes, which are predicted as intron-encoded endonucleases (HYFRA_G00013577, HYFRA_G00013578, HYFRA_G00013628, HYFRA_G00013632). Two of those genes (HYFRA_G00013577 and HYFRA_G00013578) have a GIY-YIG catalytic domain (Pfam: PF01541), whereas HYFRA_G00013577 additionally shows a LAGLIDADG endonuclease (Pfam: PF00961) and cytochrome b (C-terminal)/b6/petD domain (Pfam: PF00032). The LAGLIDADG endonuclease domain is also encoded at three further genes in this cluster (HYFRA_G00013617, HYFRA_G00013629, HYFRA_G00013645).

‘GIY-YIG’ and ‘LAGLIDADG’ families belong to group I homing endonucleases (reviewed by Stoddard 2014). They are highly specific DNA cleaving enzymes catalyzing the transfer of their own sequence and of the mobile element in which they are located, often self-splicing elements as introns and inteins.

However, there is no evidence that these genes encoding for enzymes of oxidative phosphorylation and homing endonucleases are also upregulated in strain 1511.

Toxin production

There are several upregulated genes in both strains showing similarity to genes involved in toxin production of other Ascomycota. The deduced amino acid sequence of one upregulated gene identified in strain 1511 (HYFRA_G00004959, 322 aa) shows an identity of 45.7% to thioredoxin reductase GliT of *Neosartorya fumigata* (*Aspergillus fumigatus*) (AAW03299.1, 334 aa) and carries a pyridine nucleotide-disulfide oxidoreductase domain (Pfam: PF07992). Another upregulated gene (HYFRA_G00010095, 587 aa) in strain 1431, has a major facilitator superfamily domain (Pfam: PF07690) and shows 35% identity to major facilitator gliotoxin efflux transporter GliA of *N. fumigata* (AAW03302.1, 542 aa) but also to efflux pump encoding gene AflT of *A. parasiticus* (36.5% identity).

In *A. fumigatus* *gliT* is part of a gene cluster for the biosynthesis of the epipolythiodioxopiperazine (ETP) gliotoxin and catalyzes the closure of disulfide bond of dithiol gliotoxin to final gliotoxin (Scharf et al. 2010; Schrettl et al. 2010). It also has an important role in self-protection as its deletion leads to disruption of gliotoxin release and an increased sensitivity to exogenous gliotoxin. Like the putative homologue in *H. fraxineus* it has a pyridine nucleotide-disulfide oxidoreductase domain (Pfam: PF07992). Gliotoxin is subsequently effluxed by GliA (Wang et al. 2014).

Gliotoxin is the best-known representative of the class of ETPs and is shown to have immunosuppressive features (reviewed by Ye et al. 2021). The complete gene cluster for biosynthesis of gliotoxin in *Aspergillus fumigatus* originally comprises 12 genes encoding for a two modular non-ribosomal peptide synthase (*gliP*), a glutathione *S*-transferase (*gliG*), and a hypothetical protein (*gliK*) shown to encode for a γ -glutamate cyclotransferase by Scharf et al. 2013, dipeptidase (*gliJ*), an 1-aminocyclopropane-1-carboxylic acid synthase (ACCS) (*gliI*), thioredoxin reductase (*gliT*), an O-methyl transferase (*gliM*), a methyl transferase (*gliN*), cytochrome P450 monooxygenases (*gliC* and *gliF*), major facilitator transporter (*gliA*), a zinc finger transcriptional regulator (*gliZ*) (Gardiner and Howlett 2005) as well as *gliH*, a unknown protein adjacent to the gliotoxin cluster (Schrettl et al. 2010). The detailed function of the proteins has already been reviewed elsewhere (Scharf et al. 2012, 2016; Dolan et al. 2015; Downes et al. 2023). For the proteins described to belong to the gliotoxin biosynthesis pathway putative homologs in *H. fraxineus* had been searched (Table 4). Based on similarity and domain structure, a putative homologue had been found for all proteins. For GliP, GliZ, GliI, GliC, GliG, and GliM the putative homologue protein hit showed low e-values ($< 10^{-20}$) and/or minimal identity (below 30%). However, the corresponding Pfam family/domain entry could be

found in all potential homologous proteins, except for GliK of *A. fumigatus* (AAW03303.1). But GliK it shares the same Interpro homologous superfamily (Gamma-glutamyl cyclotransferase-like superfamily; IPR036568) like its putative homologue in *H. fraxineus* (HYFRA_T00006955). For GliH (EAL88826.2) of *A. fumigatus* no homologous protein can be identified (data not shown).

Since the description of the biosynthetic gene cluster in *A. fumigatus* (Gardiner and Howlett 2005), two more transcription factors have been reported. One is the transcription factor GipA (XP_747886.1), which has two C₂H₂ zinc finger binding domains (Schoberle et al. 2014). In *H. fraxineus* there is a putative homologue protein encoded by a protein of unknown function HYFRA_T00002564 (BLASTP identities = 96/196 (49%), e-value = 4e-49) also showing two zinc finger, C₂H₂ type domains (Pfam: PF00096).

The other transcription factor is RglT of *A. fumigatus* (B0XQ41.1) (Ries et al. 2020), which has a BLASTP hit at HYFRA_T00002891 (annotated as protein of unknown function) with an identity of 92/287 (32%) and an e-value of 9e-25. They have a common fungal Zn(2)-Cys(6) binuclear cluster domain (Pfam: PF00172). However, both genes are not upregulated in our strains grown in *F. excelsior* supplemented media compared to *F. mandhurica* supplemented or control media.

Among the upregulated genes, there are two for strain 1431 and three for 1511 which are similar to genes belonging to the gene clusters of the fungal metabolites aflatoxin (*A. parasiticus*) and hypothemycin (*Hypomyces subiculosus*). In strain 1431, one of these genes is HYFRA_G00005709 encoding a predicted cytochrome p450 domain (Pfam: PF00067). As best BLAST hit against UniProt KB SwissPro database it shows an amino acid identity of 37% (e-value 6.4e-36) to cytochrome P450 monooxygenase *hpm1* of *H. subiculosus* (*Nectria subiculosa*) (B3FWR7.1), which is part of the gene cluster that mediates the biosynthesis of hypothemycin. However, it is shorter (170 aa) than Hpm1 (502 aa) and lacks a predicted transmembrane region. Compared to *A. parasiticus* aflatoxin cluster it shows 35% identity (e-value 3e-29) to averantin hydroxylase, a cytochrome P450 monooxygenase (AflG, AAS66008.1), and 35% identity (e-value 5e-29) to AflL (AAS66013.1), a versicolorin B desaturase. In *A. parasiticus*, AflG is catalyzing the conversion of averantin to 5'-hydroxyaverantin and *aflL* the conversion of versicolorin B to versicolorin A (Yu et al. 2004). Also, in comparison to AflG (495 aa) and AflL (500 aa) of *A. parasiticus* it is shorter and lacks a transmembrane region at N-terminus.

The second upregulated gene in strain 1431 (HYFRA_G00010188, 392 aa) encodes an O-methyltransferase domain (Pfam: PF00891), and compared against UniProt KB SwissPro database, it shows an 51% amino acid identity (e-value

Table 4 BLASTP results of gliotoxin gene cluster proteins of *Aspergillus fumigatus* (AY838877.1) and predicted homologs in *Hymenoscyphus fraxineus*, incl. predicted function, length (aa), Pfam hits, e-values, and identities

Protein	Predicted function	<i>A. fumigatus</i> protein accession number	Length (aa)	Pfam hit	<i>H. fraxineus</i> Protein	Length (aa)	Pfam hit	E-value	Identity
GliT	Thioredoxin reductase	AAW03299.1	334	PF07992: Pyridine nucleotide-disulfide oxidoreductase	HYFRA_ T00004959*	322	PF07992: Pyridine nucleotide-disulfide oxidoreductase	2e-97	143/313 (46%)
GliF	Cytochrome P450 monooxygenase	AAW03300.1	506	PF00067: Cytochrome P450	HYFRA_ T00006606	520	PF00067: Cytochrome P450	7e-63	164/511 (32%)
GliN	N-methyltransferase	AAW03301.1	284	PF13489: Methyltransferase domain	HYFRA_ T00009774	295	PF13489: Methyltransferase domain	3e-38	85/272 (31%)
GliA	MFS gliotoxin efflux transporter	AAW03302.1	542	PF07690: Major Facilitator Superfamily	HYFRA_ T00000080	561	PF07690: Major Facilitator Superfamily	4e-180	259/546 (47%)
GliK	Gamma-glutamyl cyclotransferase	AAW03303.1	273	/	HYFRA_ T00006955	342	PF13772: AIG2-like family	3e-31	86/279 (31%)
GliG	Glutathione-S-transferase	AAW03304.1	257	PF13409: Glutathione-S-transferase, N-terminal domain PF00043: Glutathione-S-transferase, C-terminal domain	HYFRA_ T00011011	273	PF02798: Glutathione-S-transferase, N-terminal domain PF00043: Glutathione-S-transferase, C-terminal domain	2e-18	61/204 (30%)
GliM	O-methyltransferase	AAW03305.1	439	PF00891: O-methyltransferase domain	HYFRA_ T00003247	775	PF00891: O-methyltransferase domain PF13489: Methyltransferase domain	2e-18	91/367 (25%)
GliC	Cytochrome P450 monooxygenase	AAW03306.1	512	PF00067: Cytochrome P450	HYFRA_ T00008440	499	PF00067: Cytochrome P450	4e-13	48/186 (26%)
GliP	Non-ribosomal peptide synthetase	AAW03307.1	2,125	PF00550: Phosphopantetheine attachment site PF00668: Condensation domain PF00501: AMP-binding enzyme	HYFRA_ T00014072	2967	PF00550: Phosphopantetheine attachment site PF00668: Condensation domain PF13193: AMP-binding enzyme C-terminal domain PF00501: AMP-binding enzyme	3e-108	541/2240 (24%)
GliJ	Dipeptidase	AAW03308.1	392	PF01244: Membrane dipeptidase (Peptidase family M19)	HYFRA_ T00003816	482	PF01244: Membrane dipeptidase (Peptidase family M19)	2e-88	161/408 (39%)
GliI	Probable aminotransferase	AAW03309.1	454	PF00155: Aminotransferase class I and II	HYFRA_ T00008054	479	PF00155: Aminotransferase class I and II	2e-08	57/184 (31%)
GliZ	C6 finger domain transcription factor	AAW03310.1	470	PF00172: Fungal Zn(2)-Cys(6) binuclear cluster domain	HYFRA_ T00014036	702	PF04082: Fungal-specific transcription factor domain PF00172: Fungal Zn(2)-Cys(6) binuclear cluster domain	1e-08	28/85 (33%)

*Upregulated in strain 1511

5.5e-130) to O-methyltransferase PenC (A0A1B2CTA7, 398 aa) of *Penicillium thymicola*. Compared to the hypothemycin gene cluster of *H. subiculosus* it shows 29% identity (e-value 7e-35) to O-methyltransferase Hpm5 (ACD39764.1). Compared to *A. parasiticus* aflatoxin cluster it has 28% identity (e-value 2e-51) to AflO, a demethylsterigmatocystin 6-O-methyltransferase (AAS66016.1, Q9UQY0; 386 aa)

and 25% identity (e-value 2e-28) to AflP, a sterigmatocystin 8-O-methyltransferase (AAS66017.1, 418 aa). All proteins (PenC, Hpm5, AflO, and AflP) have a O-methyltransferase domain (Pfam: PF00891) like HYFRA_G00010188. PenC has a rather unknown function in secondary metabolite biosynthesis of the alkaloid penigequinolone, whereas Hpm5 in interaction with the FAD-binding monooxygenase Hpm7

and cytochrome P450 monooxygenase Hpm1 is involved in biosynthesis (conversion of *trans*-7',8'-dehydrozearalenol (DHZ) to aigialomycin C) of hypothemycin (Reeves et al. 2008).

The protein AfIO (syn: OmtB) is described to be responsible for the conversion of demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and of dihydrodemethylsterigmatocystin (DMDHST) to dihydrosterigmatocystin (DHST) (Yabe et al. 1989, 1998; Motomura et al. 1999). AfIP (syn: OmtA) catalyzes the next step after OmtB, the conversion of both sterigmatocystin (ST) and dihydrosterigmatocystin (DHST) to O-methylsterigmatocystin (OMST) and dihydro-O-methylsterigmatocystin (DHOMST) (Yabe et al. 1989; Yu et al. 1995). Sterigmatocystin is a stable metabolite produced during aflatoxin B1 production and described to have cytotoxic effects in animals (reviewed by Zingales et al. 2020).

In strain 1511, there are two other upregulated genes encoding an O-methyltransferase domain (Pfam: PF00891). One is HYFRA_G00007274 (396 aa), which has best similarity to AfIO (OmtB) demethylsterigmatocystin 6-O-methyltransferase of *Aspergillus flavus* (Q9P900, 386 aa) with 34% identity (e-value of 5.9e-50). The second-best hit is AfIO of *A. parasiticus* (Q9UQY0, 386 aa) with an identity of 34% (e-value 2.3e-48). Both hits have the same O-methyltransferase domain (Pfam: PF00891) like the query. The other upregulated gene (HYFRA_G00003929, 422 aa) shows best similarity to grayanic acid biosynthesis cluster O-methyltransferase (46% identity; e-value 4.4e-121; E9KMQ4). Grayanic acid is the only known secondary metabolite known in *Cladonia grayi* (Armaleo et al. 2011). An additional BLAST of the amino acid sequence also shows that this gene is the best hit in *H. fraxineus* for AfIP (OmtA) sterigmatocystin 8-O-methyltransferase (AAS66017.1, 418 aa) of *A. parasiticus* aflatoxin cluster (32% identity, e-value 9e-40). Like the upregulated predicted O-methyltransferase (HYFRA_G00010188) in strain 1431 both upregulated genes in 1511 show deduced amino acid similarity to O-methyltransferase Hpm5 (ACD39764.1) of *H. subiculosus* (identity of 26%; e-value 2e-28 for HYFRA_T00007274 and e-value 9e-14 for HYFRA_T00003929).

Another product of an upregulated gene HYFRA_G00013707 (255 aa) is similar to norsolorinic acid ketoreductase of *Dothistroma septosporum* (Nor1, M2Y1A3, 268 aa) with 44.5% identity (e-value 3e-56) and *A. parasiticus* (AfID, AAS66005, 271 aa) with 43% identity (e-value 5e-60). Both hits are having the same short chain dehydrogenase domain (Pfam: PF00106) like the query. Norsolorinic acid ketoreductase (afID) catalyzes the dehydration of norsolorinic acid to form (1'S)-averantin in aflatoxin biosynthesis pathway of *A. parasiticus* (Zhou and Linz 1999). There is

no similarity to any gene product of hypothemycin cluster of *H. subiculosus*.

Apart from the upregulated genes, there are more genes encoded in the genome of *H. fraxineus* showing similarity to genes of the aflatoxin biosynthesis cluster of *A. parasiticus* (Table 5) and hypothemycin biosynthesis cluster of *H. subiculosus* (Table 6).

In strain 1511 there are more upregulated genes, showing similarity to genes of known toxin clusters. Two upregulated genes show similarity to genes for depudecin synthesis (DEP cluster) in *Alternaria brassicicola* (Wight et al. 2009). Depudecin is a polyketide that acts as a histone deacetylase (HDAC) inhibitor (Kwon et al. 1998) and contributes to virulence of *A. brassicicola* (Wight et al. 2009). This cluster consists of six genes (*DEP1–DEP6*), including four genes likely coding for enzymes that catalyze the synthesis of secondary metabolite, one gene encoding a MFS transporter (DEP3) and a gene that encodes a transcription factor (DEP6).

DEP5 is described to be the central enzyme in depudecin biosynthesis and belongs to type I reducing polyketide synthase. DEP2 and DEP4 are both predicted to encode monooxygenases. Whereas DEP2 belongs to class A and might be responsible for the epoxidations or hydroxylations of depudecin, DEP4 is of class B and likely can catalyze epoxidation. DEP1 is a protein with unknown function in depudecin biosynthesis.

The amino acid sequence of upregulated gene HYFRA_G00013234 (566 aa) is similar to FAD-dependent monooxygenase DEP4 of *A. brassicicola* (ACZ57547.1, 581 aa) with 50% identity. It has a pyridine nucleotide-disulfide oxidoreductase domain (Pfam: PF13738) like DEP4.

The amino acid sequence of the other upregulated gene (HYFRA_G00013226; 571 aa) and the DEP3 MFS transporter (ACZ57546.1) of depudecin cluster share 52% identity. Both proteins are predicted to belonging to the major facilitator superfamily of transporters (Pfam: PF07690) and having several predicted transmembrane domains.

Also, for the other proteins of *A. brassicicola* depudecin cluster a homologue sequence can be identified in *H. fraxineus* and amino acid sequences shared between 28 and 53% identity (Table 7).

DEP1, the protein with unknown function in depudecin biosynthesis, shows 31% identity to HYFRA_T00013233. Both proteins have no detectable conserved domains, but several predicted transmembrane regions. DEP2 monooxygenase shows 37% identity to HYFRA_T00013230, sharing a conserved FAD-binding domain (Pfam: PF01494).

DEP5 (ACZ57548.1) polyketide synthase shows 53% identity to HYFRA_T00013232. They share six predicted Pfam domains (PF02801, PF00698, PF16197, PF08659, PF14765, PF00109); however, two domains predicted

Table 5 BLASTP results of aflatoxin gene cluster of *Aspergillus parasiticus* (AY371490.1) and predicted homologs in *Hymenoscyphus fraxineus*, incl. e-values and amino acid (aa) identities

Protein	Predicted function	<i>A. parasiticus</i> protein accession number	<i>H. fraxineus</i> Protein	Predicted function (based on best hit on UniProtKB Swiss- Prot Database)	E-value	Identity
AflF	Norsolorinic acid reductase B	AAS66007.1	HYFRA_T00004537	Norsolorinic acid reductase A	4e-137	204/378 (54%)
AflU	Cytochrome P450 monooxygenase	AAS66021.1	HYFRA_T00001741	Trichodiene oxygenase	3e-104	172/449 (38%)
AflT	Efflux pump	AAS66020.1	HYFRA_T00000186	Efflux pump RoqT	1e-178	259/501 (52%)
AflC	Norsolorinic acid synthase	AAS66004.1	HYFRA_T00002986	Non-reducing polyketide synthase TerA	0.0	677/1800 (38%)
AflD	Norsolorinic acid ketoreductase	AAS66005.1	HYFRA_T00013707*	Norsolorinic acid ketoreductase	5e-60	108/253 (43%)
AflA	Fatty acid synthase alpha subunit	AAS66002.1	HYFRA_T00009882	Fatty acid synthase subunit alpha	0.0	535/1279 (42%)
AflB	Fatty acid synthase beta subunit	AAS66003.1	HYFRA_T00009883	Fatty acid synthase subunit beta	0.0	775/1933 (40%)
AflR	Aflatoxin biosynthesis regulatory protein	AAS66018.1	HYFRA_T00008700	Monodictyphenone cluster transcription factor	8e-11	87/418 (21%)
AflS	Aflatoxin cluster transcriptional coactivator	AAS66019.1	HYFRA_T00004969	O-methyltransferase TpcA	1e-27	74/282 (26%)
AflH	5'-hydroxyaverantin dehydrogenase	AAS66009.1	HYFRA_T00010697	5'-hydroxyaverantin dehydrogenase	1e-44	99/269 (37%)
AflJ	Versiconal hemiacetal acetate esterase	AAS66011.1	HYFRA_T00008592	Versiconal hemiacetal acetate esterase	2e-57	115/314 (37%)
AflE	Norsolorinic acid reductase A	AAS66006.1	HYFRA_T00004537	Norsolorinic acid reductase A	3e-143	201/350 (57%)
AflM	Versicolorin reductase 1	AAS66014.1	HYFRA_T00011367	Trihydroxynaphthalene reductase	5e-120	167/254 (66%)
AflN	Cytochrome P450 monooxygenase	AAS66015.1	HYFRA_T00001366	Probable sterigmatocystin biosynthesis P450 monooxygenase StcS	1e-138	205/499 (41%)
AflG	Averantin hydroxylase	AAS66008.1	HYFRA_T00008199	Probable sterigmatocystin biosynthesis P450 monooxygenase StcF	2e-105	190/502 (38%)
AflL	Versicolorin B desaturase	AAS66013.1	HYFRA_T00012386	Isotrichodermin C-15 hydroxylase	5e-100	188/522 (36%)
AflI	Averufin oxidase A	AAS66010.1	HYFRA_T00005650	Oxidoreductase AflX	2e-38	83/238 (35%)
AflO	Demethylsterigmatocystin 6-O-methyltransferase	AAS66016.1	HYFRA_T00003308	Demethylsterigmatocystin 6-O-methyltransferase	3e-108	165/391 (42%)
AflP	Sterigmatocystin 8-O-methyltransferase	AAS66017.1	HYFRA_T00003929	O-methyltransferase GsfB	9e-40	117/370 (32%)
AflQ	O-methylsterigmatocystin oxidoreductase	AAS66031.1	HYFRA_T00000952	Cytochrome P450 monooxygenase YanC	3e-54	147/505 (29%)
AflK	Versicolorin B synthase	AAS66012.1	HYFRA_T00004247	Versicolorin B synthase	8e-179	283/607 (47%)
AflV	Cytochrome P450 monooxygenase	AAS66022.1	HYFRA_T00000422	Probable sterigmatocystin biosynthesis P450 monooxygenase STCB	5e-41	142/496 (29%)
AflW	FAD-binding monooxygenase	AAS66023.1	HYFRA_T00000717	Putative sterigmatocystin biosynthesis monooxygenase StcW	3e-129	184/467 (39%)
AflX	Oxidoreductase	AAS66024.1	HYFRA_T00005650	Aflatoxin biosynthesis protein X	3e-36	84/216 (39%)
AflY	Oxidoreductase	AAS66025.1	HYFRA_T00003635	Questin oxidase	5e-55	130/427 (30%)
NadA	NADH-dependent flavin oxidoreductase	AAS66029.1	HYFRA_T00001277	12-oxophytodienoate reductase 3	4e-19	58/170 (34%)

*Upregulated in 1511

Table 6 BLASTP results of hypothemycin gene cluster of *Hypomyces subiculosus* (EU520418.1) and predicted homologs in *Hymenoscyphus fraxineus*, incl. e-values, and amino acid (aa) identities

Protein	Predicted function	<i>H. subiculosus</i> protein accession number	<i>H. fraxineus</i> Protein	Predicted function (based on best hit on UniProtKB Swiss-Prot Database)	E-value	Identity
Hpm1	Cytochrome P450	ACD39760.1	HYFRA_T00002995	Cytochrome P450 monooxygenase Hpm1	2e-170	228/321 (71%)
Hpm2	Glutathione-S-transferase	ACD39761.1	HYFRA_T00002993	Glutathione-S-transferase Hpm2	2e-109	148/210 (70%)
Hpm3	Non-reducing polyketide synthase	ACD39762.1	HYFRA_T00002986	Non-reducing polyketide synthase TerA	0.0	543/1747 (31%)
Hpm4	Hypothetical protein	ACD39763.1	HYFRA_T00006797	Protein of unknown function	0.56	30/105 (29%)
Hpm5	O-methyltransferase	ACD39764.1	HYFRA_T00001196	O-methyltransferase Hpm5	3e-132	192/397 (48%)
Hpm6	MFS transporter	ACD39765.1	HYFRA_T00000186	Efflux pump RoqT	5e-169	262/551 (48%)
Hpm7	Flavin-dependent monooxygenase	ACD39766.1	HYFRA_T00012808	Ketocytochalsin monooxygenase	2e-165	252/601 (42%)
Hpm8	Reducing polyketide synthase	ACD39767.1	HYFRA_T00010827	Reducing polyketide synthase FUB1	0.0	781/2419 (32%)
Hpm9	Alcohol oxidase	ACD39768.1	HYFRA_T00001927	FAD-linked oxidoreductase PatO	6e-75	178/583 (31%)
Unknown	Oxidoreductase	ACD39769.1	HYFRA_T00010869	Probable ferric reductase transmembrane component	5e-40	106/318 (33%)

in *H. fraxineus* protein are missing in DEP5 of *A. brassicicola*. These are an alcohol dehydrogenase GroES-like domain (Pfam: PF08240) and Phosphopantetheine attachment site domain (Pfam: PF00550). The regulatory protein DEP6 (ACZ57549.1) exhibits 28% identity to HYFRA_T00013229, and both proteins share the same domains, a fungal Zn(2)-Cys(6) binuclear cluster domain (Pfam: PF00172) and a fungal-specific transcription factor domain (Pfam: PF04082). A BLAST of the identified *H. fraxineus* protein sequences against UniProtKB Swiss-Prot database shows that they are having best hits on proteins of depudecin cluster of *A. brassicicola* and *Fusarium langsethiae*.

In *H. fraxineus* the genes are located in in close proximity at scaffold 62 but exhibited gene arrangement (Fig. 2). The genes are flanked by HYFRA_T00013225 and HYFRA_T00013235. HYFRA_T00013225 is a predicted transmembrane protein, showing similarity to H(+)/Cl(-) exchange transporters and belongs to voltage-gated chloride channel family (Pfam: PF00654). HYFRA_T00013235 is a protein of unknown function. There are three more coding sequences located between the identified homologous genes which cannot be assigned to a function: HYFRA_T00013227 belongs to a protein family of unknown function (DUF3237), HYFRA_T00013228 has a predicted signal peptide region, and HYFRA_T00013231 has a NAD(P)H-binding domain (Pfam: PF13460). Beside HYFRA_G00013234 (predicted as DEP4) and HYFRA_G00013226 (predicted as DEP3), also HYFRA_G00013227 (unknown function) is upregulated in strain 1511.

In strain 1431 none of those genes is predicted as upregulated during growth on *F. excelsior* compared to *F. mandshurica* and control media.

Another upregulated gene identified in strain 1511 is HYFRA_G00011677 (393 aa). It has a similarity to several trans-enoyl reductases, like ACTTS2 in *Alternaria alternata* (BAJ14523.1; BAJ09790.1; 343 aa) with 41.1% identity, e-value 2.6e-82. They share a predicted zinc-binding dehydrogenase domain (Pfam: PF00107) and belong to trans-enoyl reductase-like family (IPR047122). In *A. alternata* tangerine pathotypes this protein is essential for ACT-toxin biosynthesis and pathogenicity (Kohmoto et al. 1993; Ajiro et al. 2010).

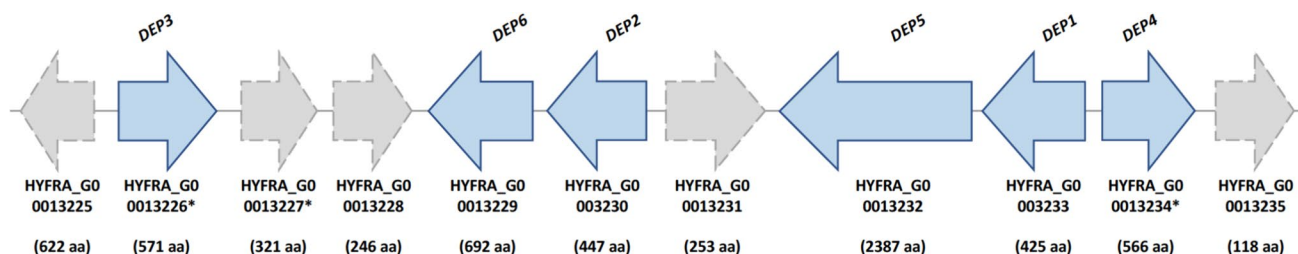
Identification of differentially expressed genes involved in pathogenicity

We also hypothesized that better growth conditions in a susceptible host like *F. excelsior* go along with the higher expression of genes for pathogenicity in *H. fraxineus*. To identify genes with a high potential for involvement in pathogenicity, intersection of upregulated DEGs during growth in *F. excelsior* media (EX) versus *F. mandshurica* media (MA) of strain 1431 and 1511 had been analyzed. The comparison of 95 upregulated genes in EX compared to MA for isolate 1431 and 483 upregulated genes in EX compared to MA for isolate 1511 resulted in an overlapping set of four genes (Table 8; Fig. 3). Those four genes show a higher transcription rate in both strains grown in EX compared to MA.

Table 7 BLASTP results of depudecin gene cluster of *Alternaria brassicicola* (FJ977165.1) and predicted homologs in *Hymenoscyphus fraxineus*, incl. predicted function, length (aa), Pfam hits, e-values, and identities

Protein	Predicted function	<i>A. brassicicola</i> protein accession number	Length (aa)	Pfam hit	<i>H. fraxineus</i> Protein	Length (aa)	Pfam hit	E-value	Identity
DEP1	Unknown	ACZ57550.1	369	/	HYFRA_ T00013233	425	/	1e-45	117/373 (31%)
DEP2	Monoxygenase	ACZ57545.1	528	PF01494: FAD- binding domain	HYFRA_ T00013230	447	PF01494: FAD-binding domain	1e-92	157/421 (37%)
DEP3	MFS transporter	ACZ57546.1	564	PF07690: Major Facilitator Superfamily	HYFRA_ T00013226*	571	PF07690: Major Facilitator Superfamily	0.0	290/563 (52%)
DEP4	Monoxygenase	ACZ57547.1	581	PF13738: Pyridine nucleotide- disulfide oxidoreductase	HYFRA_ T00013234*	566	PF13738: Pyridine nucleotide-disulfide oxidoreductase	0.0	288/579 (50%)
DEP5	Reducing polyketide synthase	ACZ57548.1	2376	PF02801: Beta- ketoacyl synthase, C-terminal domain PF00698: Acyl transferase domain PF16197: Ketoacyl- synthetase C-terminal extension PF08659: KR domain PF14765: Polyketide synthase dehydratase PF00109: Beta- ketoacyl synthase, N-terminal domain	HYFRA_ T00013232	2387	PF08240: Alcohol dehydrogenase GroES- like domain PF02801: Beta-ketoacyl synthase, C-terminal domain PF00698: Acyl transferase domain PF16197: Ketoacyl- synthetase C-terminal extension PF08659: KR domain PF00550: Phosphopantetheine attachment site PF14765: Polyketide synthase dehydratase PF00109: Beta-ketoacyl synthase, N-terminal domain	0.0	1251/2366 (53%)
DEP6	Transcription factor	ACZ57549.1	646	PF00172: Fungal Zn(2)-Cys(6) binuclear cluster domain PF04082: Fungal-specific transcription factor domain	HYFRA_ T00013229	692	PF00172: Fungal Zn(2)- Cys(6) binuclear cluster domain PF04082: Fungal-specific transcription factor domain	1e-55	181/654 (28%)

*Upregulated in 1511

**Fig. 2** Gene order in *H. fraxineus* showing homology to depudecin cluster (DEP 1–6) of *Alternaria brassicicola* (blue, continuous outline) and genes located inside or next to the cluster (gray, dashed outline), upregulated genes in strain 1511 are marked with an asterisk

One of those four shared genes (Table 9) is HYFRA_G00008114 (579 aa), which encodes a Baeyer–Villiger monoxygenase previously assigned to *vir7* of viridiol biosynthetic gene cluster (Elfstrand et al. 2021) based on homology to *vidF* of *Nodulisporium* spp. (Wang et al.

2018). It has been demonstrated that VidF, together with VidP (esterase), and VidH (dehydrogenase), is involved in demethoxyviridin (furanosteroid) biosynthesis by catalyzing the pregnane side-chain cleavage (C17 – C20).

Table 8 Upregulated genes of *Hymenoscyphus fraxineus* isolate 1511 and 1431 grown in maltose-yeast-peptone media (MYP) supplemented with plant material from *Fraxinus excelsior* (EX) compared to MYP media supplemented with plant material from *Fraxinus mandshurica* (MA)

<i>H. fraxineus</i> isolate	Comparison group	Upregulated genes	Shared upregulated genes
1431	EX vs. MA	95	4
1511	EX vs. MA	483	

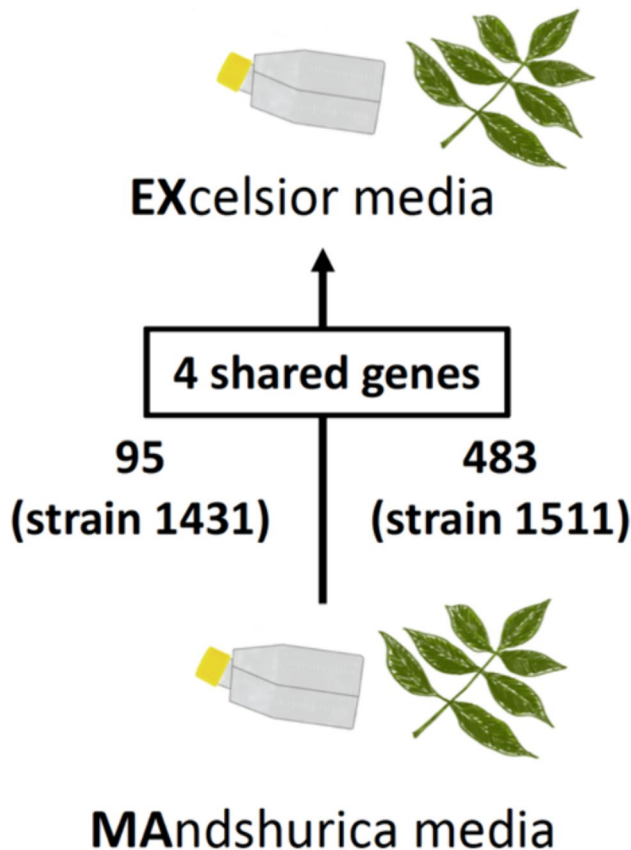


Fig. 3 Upregulated genes of *Hymenoscyphus fraxineus* isolate 1511 and 1431 grown in maltose-yeast-peptone media (MYP) supplemented with plant material from *Fraxinus excelsior* (EX) compared to MYP media supplemented with plant material *Fraxinus mandshurica* (MA)

A further gene (HYFRA_G00001588, 398 aa) encodes for a protein of unknown function, for which Phobius predicts a signal peptide indicating secretion. Pfam shows a neprosin domain (PF03080), first described in pitcher plants as peptidase in digestive fluid (Lee et al. 2016).

Another gene (HYFRA_G00009022, 577 aa) has a similar protein sequence (30.2% identity; e-value 2.4e-62)

to FAD-dependent monooxygenase DEP4 of *F. langsethiae* (A0A0M9ER62, 580 aa). It has a pyridine nucleotide-disulfide oxidoreductase domain (Pfam: PF07992), and also DEP4 protein of *F. langsethiae* belongs to the pyridine nucleotide-disulfide oxidoreductase family (Pfam: PF13738). For gene HYFRA_G00013234, upregulated in strain 1511 grown *F. excelsior* compared to *F. mandshurica* supplemented and control media, a similarity to an FAD-dependent monooxygenase DEP4 protein of *A. brassicicola* has been previously mentioned.

In the shared gene set, there is one more upregulated gene (HYFRA_G00004959; 322 aa) which is assigned to thioredoxin reductase *gliT*. It appeared already in the upregulated gene set of strain 1511 grown in *F. excelsior* compared to *F. mandshurica* supplemented and control media.

Discussion

The invasive ascomycete *H. fraxineus* is threatening *F. excelsior* populations throughout Europe. The exact mechanisms of pathogen–host interaction have not yet been elucidated. The comparison of the transcriptome during growth on media supplemented with *F. excelsior* and *F. mandshurica* leaf material performed in this study, shows that there are differentially expressed genes.

Transcriptome during growth on media supplemented with leaf material from *F. excelsior* compared to *F. mandshurica* and control media

In particular, the increased expression of genes involved in oxidative phosphorylation during growth of strain 1431 in *F. excelsior* supplemented media indicates better growth compared to *F. mandshurica* and control media. It was previously shown in Carrari et al. 2015 that growth rates were highest on *F. excelsior* supplemented media. This may be due to different or additional metabolites like carbon or nitrogen which leads to better growth. One may speculate why these genes are not upregulated in strain 1511 during growth in *F. excelsior* supplemented media. Probably strain 1511 was already in another physiological state at the timepoint of sampling, or it is missing the genes to utilize the metabolites which *F. excelsior* is providing. Also homing endonuclease genes, which are located on the same scaffold, are only predicted to be upregulated in strain 1431, but not in strain 1511.

Beside the upregulated genes for oxidative phosphorylation in strain 1431, in both strains there are genes upregulated with similarities to genes of the gliotoxin pathway, precisely a predicted *gliA* (strain 1431) and *gliT* (strain

Table 9 Shared upregulated genes of *Hymenoscyphus fraxineus* isolate 1511 and 1431 grown in maltose-yeast-peptone media (MYP) supplemented with plant material from *Fraxinus excelsior* compared to MYP media supplemented with plant material *Fraxinus mandshurica*

<i>H. fraxineus</i> gene	Length (bp)	Effector prediction	Annotation based on protein similarity (UniProtKB Swiss-Prot and Pfam) or literature
HYFRA_G00008114	1,740	No	Baeyer–Villiger monooxygenase (Cyclohexanone 1,2-monooxygenase belonging to flavin-binding monooxygenase-like family (Pfam: PF00743) assigned to <i>vir7</i> of viridiol biosynthetic gene cluster (Elfstrand et al 2021)
HYFRA_G00001588	1,617	Yes	Protein of unknown function with a neprosin domain (Pfam: PF03080)
HYFRA_G00009022	1,858	No	FAD-dependent monooxygenase DEP4 of pyridine nucleotide-disulfide oxidoreductase family (Pfam: PF13738)
HYFRA_G00004959	1,504	No	Thioredoxin reductase <i>gliT</i> / Gliotoxin biosynthesis protein with Pyridine nucleotide-disulfide oxidoreductase with a pyridine nucleotide-disulfide oxidoreductase family domain (Pfam: PF07992)

1511). *GliA* is a transporter responsible for gliotoxin efflux (Wang et al. 2014) and *GliT*, an oxidoreductase, converts between the reduced (dithiol gliotoxin) and oxidized (gliotoxin) form (Scharf et al. 2010). In *A. fumigatus* both genes are involved in self-resistance, as deletion of *gliA* and *gliT* has led to enriched intracellular gliotoxin and increased sensitivity to exogenous gliotoxin (Scharf et al. 2010; Schrettl et al. 2010; Wang et al. 2014). Beside those two genes *gliA* and *gliT*, there are further genes for gliotoxin biosynthesis and regulation described in *A. fumigatus* (Scharf et al. 2012, 2016; Dolan et al. 2015), for which a putative homologous counterpart in *H. fraxineus* was identified. The identified putative homologous genes for gliotoxin biosynthesis do not seem to be organized in a gene cluster, as it is described for *A. fumigatus* (Gardiner and Howlett 2005) and for other ascomycetes (Patron et al. 2007). However, for *Trichoderma* spp. a separation of gliotoxin biosynthetic genes is known (Bulgari et al. 2020) and also for secondary metabolite coding genes in *Fusarium* spp. (Proctor et al. 2009). So far, it is unclear how gliotoxin production is stimulated, but for *A. fumigatus* it is suggested that different factors play a role like pH, temperature, composition, and aeration of the culture media (Scharf et al. 2012).

Probably, leave substrate of *F. excelsior* is stimulating the production of a unknown toxin similar to gliotoxin in *H. fraxineus*, which acts as a virulence factor and causes damage the host plant, which could be reflected in an upregulated gene expression of genes homolog to self-resistance genes *gliT* and *gliA*.

One may consider that the primary role of those two upregulated genes is not protection against own toxins, but the defense against extracellular toxins, like it has been demonstrated for *T. virens* GV29-8 (Bulgari et al. 2020). In this case one may speculate that elevated transcription of *gliA* and *gliT* could be induced due to a toxin from another fungus that colonizes the leaves of *F. excelsior*, which withstood the autoclaving of the culture media. It is not clear if the toxin is stable during autoclaving the culture media, but it

has been previously shown that the immunosurface of a *A. fumigatus* spore—released toxin is heat stable at least 5 min at 100 °C (Mitchell et al. 1997).

Another explanation for elevated toxin production and secretion, probably indicated by elevated *gliA* and *gliT* transcripts, is the prevention of side effects of intracellular oxidative stress for *A. fumigatus* (discussed in Schrettl et al. 2010; Owens et al. 2014). In this case, gliotoxin would act as electron acceptor and/or would help as buffer against endogenous and exogenous oxidants and is subsequently secreted. The cytotoxic effects on host cells therefore would be only a side effect. Probably, the increased growth of *H. fraxineus* in *F. excelsior* supplemented media leads to oxidative stress and stimulates the production of a molecule as part as an intracellular antioxidant defense system which unintentionally acts as toxin.

The comparison with the gene clusters for aflatoxin (*A. parasiticus*) and hypothemycin (*H. subiculosus*) shows that several genes with high similarities to genes involved in toxin production are encoded in the genome. Some of them are upregulated during growth on *F. excelsior* supplemented media compared to *F. mandshurica* and control. Those comprise genes with an O-methyltransferase domain (Pfam: PF00891) (HYFRA_G00010188 in strain 1431; HYFRA_G00007274 and HYFRA_G00003929 in strain 1511), but also one gene with a predicted cytochrome p450 domain (Pfam: PF00067) (HYFRA_G00005709 in strain 1431) and a short-chain dehydrogenase domain (Pfam: PF00106) (HYFRA_G00013707 in 1511). Like for gliotoxin, the growth conditions, especially pH and nutrients (especially of carbon and nitrogen), are described as parameter influencing aflatoxin production (Caceres et al. 2020). Oxidative stress is also proposed to induce aflatoxin production. Not only final aflatoxin but also intermediate compounds like versicolorin A could have strong cytotoxic effects, as shown for human intestinal cells (Gauthier et al. 2020). It has been shown previously that in *H. fraxineus* compared to *H. albidus* there are

more homologs of versicolorin B synthase genes (Elfstrand et al. 2021).

The depudecin gene cluster is widely found within the Ascomycota, but it is discontinuously distributed (Reynolds et al. 2017). *A. brassicicola* depudecin is leading to lesions on cabbage leaves (*Brassica oleracea*). For all genes of depudecin cluster in *A. brassicicola* homologous genes organized in a cluster can be identified in *H. fraxineus*. One may consider that these genes are responsible for a depudecin-like secondary metabolite in *H. fraxineus*. Depudecin of *A. brassicicola* turned out to be a minor virulence factor (Wight et al. 2009), but there are also other HDAC inhibitors, like HC-toxin of *C. carbonum*, acting as strong virulence factor on maize (Brosch et al. 1995).

In summary, in *H. fraxineus* strains 1511 and 1431 grown in *F. excelsior* supplemented media compared to *F. mandshurica* and control, there are upregulated genes predicted to have similar functions and pathways, especially for gliotoxin, hypothemycin and aflatoxin, despite that there are no shared genes between both datasets.

However, genes predicted to be involved in oxidative phosphorylation as well as endonucleases are only predicted to be upregulated in 1431, and genes of depudecin pathway only in 1511. The different sample preparation methods of three pooled samples (strain 1511) versus triplicates (strain 1431), could account for a certain amount of differentially expressed genes predicted for the strains despite the same treatment. This is reflected in the DEGs set of strain 1431 grown in *F. excelsior* compared to *F. mandshurica* media (126 DEGs) in contrast to the same comparison group for 1511 (1,273 DEGs), which could be due to a triplicate outlier of strain 1431 grown in *F. excelsior* which can be seen in principal component analysis figure (not shown). The comparison results on the transcriptome of the two strains are also limited to the respective time point of sampling. Thus it may not consider the different growth rates of the strains and the physiological state e. g. exponential growth phase probably reflected by the oxidative phosphorylation. Beside those thoughts, it could be also that the strains have a unique transcriptome profile.

Shared upregulated genes between both strains during growth on media supplemented with leaf material from *F. excelsior* compared to *F. mandshurica*

Four genes are upregulated in both strains during growth on *F. excelsior* supplemented media, compared to *F. mandshurica*.

One gene encodes a protein with an unknown function (HYFRA_G00001588). It has a predicted neprosin domain (Pfam: PF03080) and a signal peptide. Neprosin is described as a protease with prolyl endopeptidase (PEP) activity (Lee

et al. 2016). The exact function of this protein in *H. fraxineus* is not clear. As it possesses a signal peptide, it likely has an extracellular peptide cleavage activity.

Another gene (HYFRA_G00009022) shows similarity to FAD-dependent monooxygenase DEP4 (A0A0M9ER62) of *F. langsethiae*. In *Alternaria brassicicola* this gene belongs to the depudecin gene cluster, an inhibitor of histone deacetylase (Wight et al. 2009). The exact function of this protein in *H. fraxineus* is not clear, but as discussed previously it is probably involved in production of a toxin similar to depudecin.

The upregulated gene HYFRA_G00004959 has an identity to thioredoxin reductase *gliT* of gliotoxin cluster of *A. fumigatus*, which has already been discussed previously. In the upregulated gene set of *F. excelsior* supplemented media compared to *F. mandshurica* and control media it was only upregulated in strain 1511. However, it is predicted to be upregulated in both strains grown in *F. excelsior* supplemented media compared to *F. mandshurica*.

The upregulated gene HYFRA_G00008114 is encoding *vir7*, a Baeyer–Villiger monooxygenase, of viridiol biosynthetic gene cluster (Elfstrand et al. 2021). Viridiol can induce necrosis on ash tissues (Andersson et al. 2010; Cleary et al. 2014), but it is unlikely to be the main responsible agent for ash dieback symptoms, as it is also produced by the non-phytopathogenic *H. albidus* and higher concentrations do not seem to come with higher virulence (Junker et al. 2014). Interestingly, for both strains this gene is only upregulated during growth in *F. excelsior* compared to *F. mandshurica*, but not control media. This result may be interpreted with an acceleration of foliage senescence and suppression of other competing microbial organisms. Such interaction is largely harmless for the host at the end of the growing season in Asia but may promote disease in Europe. In strain 1511 there are additional genes of this cluster predicted to be upregulated during growth in *F. excelsior* media compared to *F. mandshurica*: *vir4* (HYFRA_G00008111), *vir6* (HYFRA_G00008113), *vir8* (HYFRA_G00008115), and *vir10* (HYFRA_G00008117).

Hymenosetin is another described secondary metabolite described for *H. fraxineus* (Halecker et al. 2014). It is discussed to have antimicrobial effects to provide an advantage in terms of substrate availability, but shown to have no or only moderate cytotoxic effects. Genes belonging to the biosynthetic pathway of hymenosetin, do not seem to be upregulated in our analyzed datasets.

It has been shown that the defense mechanisms in *F. mandshurica* against *H. fraxineus* take place in the leaves (Gross and Holdenrieder 2015). This suggests that the leaves of the different ash species have an influence on the growth and virulence mechanisms and therefore gene expression profile of *H. fraxineus*. This is reflected by our results, which show differentially expressed genes between growth

in media supplemented with *F. excelsior* and *F. mandshurica* leaf material. It is very likely that some of the higher expressed genes are involved in virulence mechanisms.

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Author contributions MK and CZ conceived and designed the study; CZ performed the experiments and analyzed the data, while both authors contributed to the interpretation of the results; CZ prepared the initial manuscript; both authors approved the manuscript.

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Data availability Raw reads have been submitted to the Sequence Read Archive (SRA) under accession numbers SRR25865483—SRR25865494. The project has been deposited under BioProject accession number PRJNA1011055 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1011055>).

Declarations

Conflict of interest The authors declare no conflict of interest.

Consent to participate Approved by all authors.

Consent for publication All authors declare consent of publication.

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