Genomes of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus* Encode Surprisingly Large Cell Wall Degrading Potential, Balancing Saprotrophic and Necrotrophic Signatures

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Abstract

In Europe, an epidemic is currently occurring on common ash (*Fraxinus excelsior*). The disease, commonly known as ash dieback, is the result of a biological invasion by the causal Helotialean fungus *Hymenoscyphus fraxineus* Baral, Queloz, Hosoya. This study describes the genomes of *H. fraxineus* and *H. albidus,* a native non-pathogenic sister species to *H. fraxineus.* The *Hymenoscyphus* sp. genomes harbour similar and extensive Cell Wall Active Enzyme (CAZYme) repertoires, they appear better at degrading cellulose than e.g. *Botrytis* but has similar pectin-degrading capacities. *In planta*, the pathogenic *H. fraxineus* showed higher gene expression than *H. albidus* of two of the pectin degrading enzymes, consistent with a higher disruption of primary cell walls and possibly leading to a stronger host reaction. Based on SignalP and Phobious annotations, we identified 2160 and 2006 secreted genes in *H. fraxineus* and *H. albidus*, respectively. This is almost twice as many as for most other Helotialean fungi. Two small secreted proteins were transcribed in *H. fraxineus*, one being a cerato-platinin like protein with a putative role in pathogenicity. No small secreted proteins were detected in the *H. albidus* transcriptome. It has been suggested that fungal metallopeptidases, can target and degrade non-structural defense proteins *in planta.* We found that the *Hymenoscyphus* genomes encode more metallopeptidases than other Helotialean species. In conclusion, the prolonged saprotrophic growth phase on shed ash leaves of *H. fraxineus* and *H. albidus* has probably shaped the genomes. Both genomes are highly similar and have CAZYme profiles similar to saprotrophic fungi. The relatively small differences between the two *Hymenoscyphus* spp. in gene expression are likely indicative of their differential interaction patterns with the host tree *F. excelsior.*

Keywords: Ash dieback, *Hymenoscyphus fraxineus, Hymenoscyphus albidus, Fraxinus excelsior*, cell wall degrading enzymes, small secreted proteins, metalloproteinases, invasive species

Introduction

European ash (*Fraxinus excelsior*) has been the subject of a sever die back during the last decades (Kowalski 2006, Bakys et al. 2009, Gross et al. 2014). The disease is caused by an introduced pathogen *Hymenoscyphus fraxineus* that in its origin is an endophyte or weakly pathogenic fungus (Cleary et al. 2016). The life cycle includes spore infection on leaves, or occasionally bark tissue, followed by growth via the petiole into twigs (Gross et al. 2014). Unchecked growth in the inner bark and xylem result in dieback of the crown, which ultimately can lead to mortality of the whole tree. Sporulation occurs on fallen leaf rachises typically the year after initial leaf infection and spores are produced in large amounts particularly after rainy periods in late summer. In Europe, a close relative *H.albidus* has long been present on ash leaves and fallen rachises without pathogenic growth in host woody tissue.

Sequencing the whole genome of an organism is invaluable for its comprehensive molecular characterization and has been drastically facilitated by the advent of high-throughput sequencing techniques. Important progress is currently made in our understanding of the genomic organization and the genetic repertoire of a wide range of fungi through the release and subsequent mining of genome sequences. Evolutionary and bioinformatic analyses of pathogen and non-pathogen genomes represent novel ways with which to decipher mechanisms controlling associations with the host tree and shed light on the acquisition of the genetic toolkit during fungal evolution.

The rapid spread of *H. fraxineus* following the introduction into Europe and the high susceptibility of *Fraxinus excelsior* poses the question as to if there are any specific parts of the genetic setup in the fungus that could explain its success. In order to shed light on this we have sequenced and assembled the genomes of *H. fraxineus* and *H. albidus* and performed comparative investigations of their genetic content. With the aim of putting this information into further perspective we also included a set of previously sequenced closely related ascomycete genomes of species with diverse ecological strategies. All *Hymenoscyphus* genomes were *de novo* assembled and genes in all studied genomes were annotated using the same methodology. Here we report on the gene content of cell wall degrading enzymes secreted proteins and metalloproteases.

Materials and Methods

Cultivation of fungal isolates and DNA extraction for genome sequencing

The *Hymenoscyphus* isolates (*H. fraxineus* isolate nf4 and *H. albidus* isolate 111/1/4) were cultivated in in liquid malt extract 1.75% and 0.25% peptone at 22°C for 4 weeks. DNA was extracted from mycelium samples according to CTAB/chloroform methods and samples were subsequently treated with RNase A (Sigma). After quality check, DNA was sent to SciLifeLab, Uppsala, Sweden, where sequencing libraries with a target insert size of 500bp were prepared. The libraries were sequenced using a paired end protocol with 100 sequencing cycles from each end on an Illumina HiSeq 2000 sequencer. Moreover, mate pair sequencing was done on an ABI SOLiD sequencing instrument (Life Technologies) using Exact Call Chemistry. For *H. fraxineus* two libraries with insert sizes of about 3 kb and 8 kb were prepared and sequenced, and for *H. albidus* a single library with an insert size of about 3 kb was prepared and sequenced. The mate pair libraries were sequenced with 60bp from each end (Elfstrand et al. in prep).

All data deposition numbers are available on request from Mikael Brandström Durling (Mikael.Durling@slu.se).

Annotation and evolution of carbohydrate active enzymes

To annotate all carbohydrate active enzymes (CAZymes) in the genomes, we used HMMER 3.0 (Finn et al. 2011) with Hidden Markov Models (HMMs) from dbCAN (Yin et al. 2012). The sizes and evolution of the different CAZyme families were compared using CAFE, following the same procedure as for the more general gene family evolution analysis.

Annotation of short secreted proteins (SSPs)

We defined Short Secreted Proteins (SSPs) as proteins with at most 150 amino acids, and having a signal peptide for secretion present. We also required that the protein did not contain any predicted trans-membrane domains. Signal peptide predictions were done with SignalP (Petersen et al. 2011) and Phobius (Kall et al. 2004). Transmembrane domains were annotated with Phobius. From the set of inferred SSPs we extracted the secreted peptides, by discarding the annotated signal peptide, and used these sequences for further analysis of the secretome. Furthermore we characterized the content of conserved motifs among the secreted peptides within each species using MEME (Bailey et al. 2015) searching for motifs of 3 to 13 bp occurring zero or one time per sequence. The discovered motifs were then searched for in the peptide set where they were discovered, as well as in all other species in the study. Tests for enrichment of individual motifs were done using the Fisher's exact test.

Gene models of other Helotialean taxa and evolutionary comparisons

To enable comparative genomic analysis of the focal species in a phylogenetic framework, we used published Helotiealean fungi for comparisons; *Ascocoryne sarcoides*, *Botrytis cinerea, Glarea lozoyensis, Marsonnina brunnea* f. sp. *multigermtubi, Sclerotinia sclerotiorum* and *Sclerotinia*

borealis (Amselem et al. 2011, Gianoulis et al. 2012, Mardanov et al. 2014, Youssar et al. 2012, Zhu et al. 2012), as well as the outgroup *Blumeria graminis* (Spanu et al. 2010). Since the gene annotations of these species were produced with a variety of different annotation pipelines and vary in age, we re-annotated them with MAKER following the same procedure as we used for the *Hymenoscyphus* species and utilising the available EST or RNASeq evidence that was available in conjunction with the genome sequences.

To test for expansions and contractions among the gene families, we used CAFE version 3.1 (De Bi et al. 2006). We used default settings in CAFE and let the program estimate the underlying birth-death ration. CAFE requires an ultrametric phylogenetic tree describing the relationship between the species. We constructed a maximum likelihood tree of the species based on the genes *rpb, tef1, tub1* and *tubA* using the timetree method of MEGA4 (Tamura et al. 2007), assuming the substitution rate as a proxy for the divergence times in the tree.

Analysis of RNASeq data

Expression of fungal genes in mixed tissues was determined by RNA sequencing. RNASeq data was filtered with Nesoni to trim off adaptor sequences, and to discard low quality sequences (same parameters as for the genome sequencing) before further processing. We used the Tophat-Cufflinks-Cummerband workflow as outlined in Trapnell et al. (Trapnell et al. 2012) to estimate expression as FPKM and to test for differential expression. Tophat and Cufflinks were provided with the MAKER gene predictions to hint the alignments, and the parameters were adjusted to better fit fungal data by allowing introns of 5-5000 bp and disabling coverage based merging of transcripts.

Experimental conditions, plant material, inocula

To test *in planta* expression of selected candidate genes, inoculations were conducted on 2-year-old bare-root seedlings of *F. excelsior* (30–50 cm in height) obtained from a commercial nursery in Sweden (Cleary et al. 2013). Seedlings were grown in 20 cm diameter plastic pots filled with potting media consisting of 60% light peat sieved, 25% black peat and 15% sand (Hasselfors Garden, Örebro, Sweden) in a greenhouse with a 16 h photoperiod of 20/15°C (day/night). To prepare inocula, sterile woody plugs were added to 2-week old 2% malt extract agar cultures of both *H. fraxineus* and *H. albidus*. After 4 weeks, fully colonized woody plugs were used for inoculating plants. A minimum of eight replicate plants of *F. excelsior* were wound-inoculated with either *H. fraxineus* or *H. albidus*, and sealed with Parafilm™. Control treatments were non-wounded. Plants were harvested after 7, 14, 28, and 42 days. Stems were dissected longitudinally and the extent of necrosis measured. Phloem tissue was collected from the margin of defined lesions, frozen in liquid nitrogen and then stored at -80°C until further processing.

RNA extraction and RT-QPCR ANALYSIS

Total RNA was extracted from the phloem samples representing different time points and treatments using a standard protocol (Chang et al. 1993). Again, the RNA was treated with DNaseI (Sigma) to remove the genomic DNA. RNA quality and concentration was measured on the Bio-Analyzer 2100 using RNA Nano Chips (Agilent). Dynabeads mRNA Purification Kit (Invitrogen) was used to extract polyA+RNA. mRNA amplification was performed using the MessageAmpIII kit (Ambion). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) from purified aRNA (1µg) of either *H. fraxineus* or *H. albidus* samples taken at 7, 14, and 28 dpi with an extended RT-reaction for 50 min.

The cDNA was diluted 1:1 in deionizer water, and an aliquot of cDNA equivalent to 25 ng of aRNA was used per 20 µL of PCR reaction using SSoFast EVAGreen Supermix (Bio-Rad) with a final concentration of 0.5 µM of each primer. Primers were designed from isotig sequences using the Primer3 software (http://primer3.wi.mit.edu/) with a melting temperature (Tm) between 58°C and 60°C, and amplicon length between 95-150 bp (Table 1). The thermal-cycling condition parameters, run on a iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad), were

Table 1. Primer sequences for the gene expression analysis

Gene	Forward primer 5' -3'	Reverse primer 5' -3'
UBC	CCTCGGACTCTCCATACTCG	GATAGATTCTGGTGGTGAAGTT
RPL17	AAGCAATCAACGGATGGAAG	CGGCATAACGTCTCATAGGAACA
HYFRA_T0002081	AGCTTGGTTTCACCGAGAGA	ATCACGGTTTGGTGTGGTTT
HYFRA_T0004626	TCCCAAGATGGATCTGGAAC	AGCATCCCAAAGGTACATGC
HYFRA_T0002445	AACGTCTGGCTCTTCTTCCA	ATGGGCAGTTCACCATGACA
HYFRA_T0000350	GGAGCCGACTACTTCACCAC	ATTCTTGGGGAGGTGCTTTG
HYFRA_T0005905	TAAGGCTGCTGAGTCGTCAA	AAGTTCCGGTGTGTGAGTCC
HYFRA_T0000722	TGCTCATGCTGTCGCTACTT	CTGGGAGCTTCTGTCTCCAC

as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 58 or 60 °C for 20 s. Each run was followed by a melt curve analysis to validate the specificity of the reaction. A linear plasmid standard curve was used to measure the PCR efficiency and primer pairs with efficiency lower than 95% were discarded. Three biological replicates for each time point and two technical replicates were prepared for each sample. Transcript abundance was normalized to the reference genes *UBC* and *RPL17*. The relative expression was calculated using the delta–delta CT-method (Livak & Schmittgen 2001). Kruskal-Wallis- and Mann-Whitney U-tests were performed on the RT-qPCR data using the GraphPad Prism5 software (GraphPad Inc.).

Results and discussion

Carbohydrate active enzymes

We annotated carbohydrate active enzymes (CAZymes) in all studied genomes using the dbCAN database (Yin et al. 2012) of Hidden Markov Models. A total of 688 CAZymes in 137 gene families and 659 CAZymes in 133 gene families were found in *H. fraxineus* and *H. albidus*, respectively. At least 186 *H. fraxineus* and 183 *H. albidus* CAZymes were associated with plant cell wall degradation (PCWD) (Table 2 and 3). The proportion of PCWD CAZymes in *Hymenoscyphus* spp. (1.5% of all predicted proteins) was similar to other Helotialean fungi. The necrotrophic pathogens *S. sclerotiorum* and *B. cinerea* have been suggested to be especially suited to degrade pectin as their genome harbours many pectin-degrading, but few cellulose degrading CAZymes (Amselem et al. 2011, Blanco-Ulate et al. 2014). Our analysis suggested that *H. fraxineus, H. albidus* and *M. brunnea* possess significant pectin-degrading capacities, but also that *H. fraxineus* and *H. albidus* has a higher number of cellulose degrading enzymes than *B. cinerea*, *S. borealis*, *S. sclerotiorum* and *M. brunnea* (Table 2).

It has been argued that the number of genes encoding for cellulolytic, hemicellulolytic and pectinolytic activities in a fungal genome is a reflection of the fungal strategy for degrading plant tissues (Amselem et al. 2011, Lyu et al. 2015, Ohm et al. 2012, Zhao et al. 2014). The broad host range necrotrophic fungi *B. cinerea* and *S. sclerotiorum* have been suggested to have a higher number of gene copies and therefor a larger capacity to degrade pectin than hemicellulose or cellulose compared to other Plant Cell Wall (PCW)-degrading pathogens, while the genomes of saprotrophic fungi such as *Neurospora crassa* and *Trichoderma reesei* indicate a preference for cellulose and hemicellulose over pectin (Amselem et al. 2011). In general it can be observed, that the two Hymenoscyphus species have a reportoir of cell wall degrading enzymes that is somewhat intermediate to those of the saprotrophic and necrotrophic/hemibiotrophic members of Helotiales. The PCW-degrading enzyme profiles in the genomes of *H. albidus* and *H. fraxineus* suggest that these fungi have an obvious and very similar potential to degrade plant cell walls, similar to that of the closely related species *G. lozoyensis*. However, this PCW-degrading capacity appears to differ from the profiles of the broad range necrotrophic pathogens *B. cinerea* and *S. sclerotiorum* (Amselem et al. 2011) in that *H. albidus* and *H. fraxineus* have a larger arsenal of PCWD enzymes and more potential to utilize cellulose and hemicelluloses as a carbon source, reminiscent of the PCW-degrading enzyme profiles described for several saprotrophs (Amselem et al. 2011).

In our analysis, we tested for expansion or contraction of specific CAZyme families using CAFE, assuming the same birth-death rate of family members as was estimated from the analysis of the full proteome gene families. We found four CAZyme families with significant expansions and one contraction in the ancestor to *Hymenoscyphus* (Figure 1). The higher number of cellulose degra-

Table 2. Summary table of genome content of cell wall degrading enzymes for nine Helotialean species of different ecological strategies. The number of genes and the percentage of genes out of the full gene space in the species are indicated for four categories of carbohydrate substrate

		CAZymes per PCW component								
	Trophic strategy	Cellulose		Hemicellulose		Hemicellulose w. pectic sidechains		Pectin		To-tal
		N	*%*	N	*%*	N	*%*	N	*%*	
A. sarcoides	Saprotroph	20	*15*	74	*55*	18	*13*	22	*16*	134
G. lozoyensis	Saprotroph	39	*20*	99	*51*	26	*13*	31	*16*	195
H. albidus	Endophytic fungus	30	*16*	92	*50*	22	*12*	39	*21*	183
H. fraxineus	Necrotropic pathogen	31	*17*	95	*51*	21	*11*	39	*21*	186
M. brunnea	Hemibiotrophic pathogen	22	*15*	70	*46*	21	*14*	38	*25*	151
B. cinerea	Necrotropic pathogen	22	*14*	75	*47*	17	*11*	44	*28*	158
S. borealis	Necrotropic pathogen	17	*12*	69	*50*	15	*11*	38	*27*	139
S. sclerotiorum	Necrotropic pathogen	20	*14*	71	*51*	16	*11*	33	*24*	140
*B. graminis**	Biotrophic pathogen	3	*14*	15	*71*	2	*10*	1	*5*	21

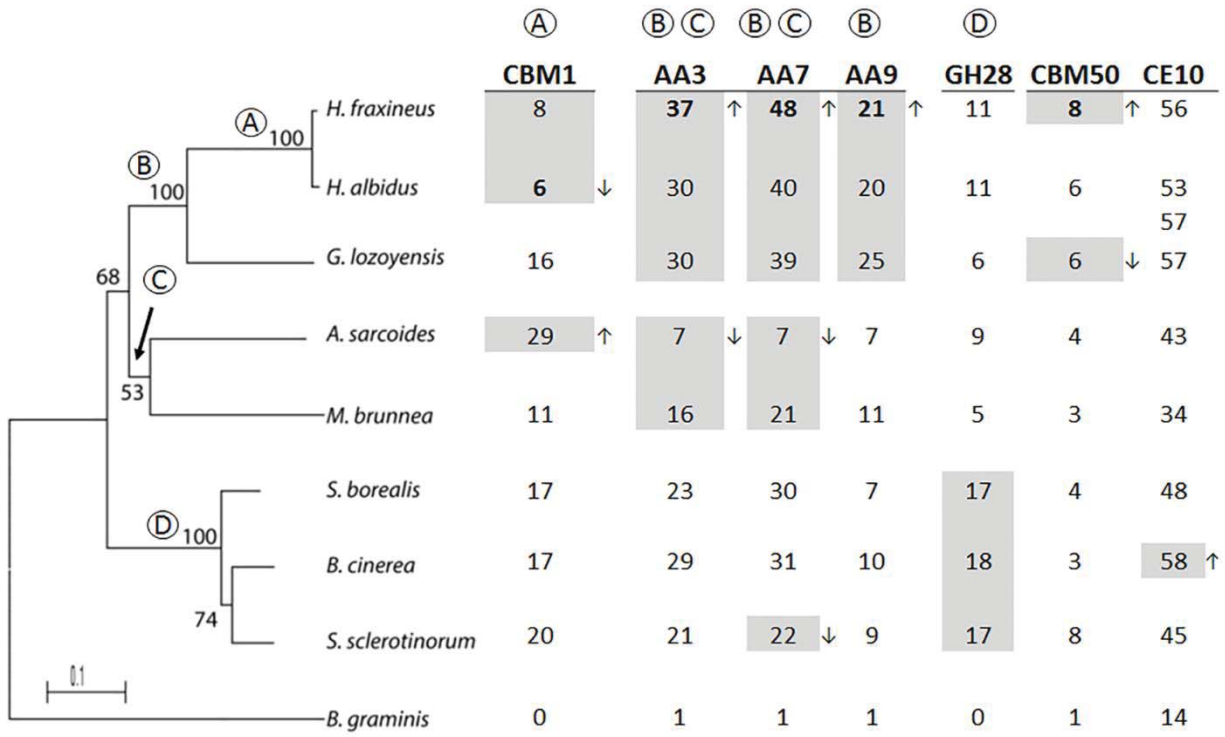

Figure 1. Expansions and contractions of CAZY gene families in the helotiales: The number of genes identified in CAZYme gene families with significant expansions and contractions in the helotiales. CAZYme gene families with significant (Viterbi *P*-value < 0.05) expansions or contractions in specific taxa or ancestral species are shaded in grey. The arrows indicate expansion (↑) or contraction (↓) for individual species along the tree. Expansion or contractions in ancestral species are indicated by encircled A, B, C and D at the node in question and above the columns of the CAZYme gene families. Numbers in bold font indicate expansions / contractions in *Hymenoscyphus*

ding enzymes in *H. fraxineus, H. albidus* and *G. lozoyensis* was traced to a significant expansion (p = 0.001) of the CAZY class auxiliary activities (AA) family 9 (formerly glycoside hydrolase (GH) family 61) in the *H. albidus*, *H. fraxineus* and *G. lozoyensis* branch (Figure 1)**.** Despite expansions of particular **c**ellulose-degrading CAZY classes in *Hymenoscyphus* spp., the number of cellulose binding domain (CBM) family 1-containg genes were significantly lower in *Hymenoscyphus* spp. compared with other Helotialean fungi (Figure 1).

Although the total numbers of predicted hemicellulose-degrading PCWD enzymes were larger in the *H. albidus*, *H. fraxineus* and *G. lozoyensis* genomes compared to other members of the Helotiales, the proportion of hemicellulose-degrading PCWD enzymes among the CAZYmes remained essentially constant, around 50%, among all Helotialean fungi **(**Table 2**).** Likewise the number of enzymes, targeting hemicellulose with pectic side chains was higher in these three species than in e.g. *B. cinerea,* although the percentual proportion was similar (Table 2). This was primarily a result of a larger GH43 family in *Hymenoscyphus* spp. and *G. lozoyensis*.

Overall, *H. albidus* and *H. fraxineus* possessed similar numbers of pectin-degrading and pectin-modifying CAZYs as the necrotrophic Helotialean fungi, although *H. albidus* and *H. fraxineus* both contained 11 GH28 genes while the closely related *G. lozoyensis* only had six. This indicates that the *H. albidus* and *H. fraxineus* have pectin-degrading potentials similar to that of *Sclerotinia* spp. and *B. cinerea*.

Gene expression was significantly higher *in planta* for *H. fraxineus* compared to *H. albidus* for two proteins involved pectin degrading activity PL3 and GH 28 (Figure 2a and b). There was no significant difference in CE 12 a gene involved in breakdown of cellulose (Figure 2c). The difference in expression of pectinolytic genes would fit well with the much higher level of damage caused by *H. fraxineus* when growing in *F. excelsior*. The enzymatic activity as such will degrade the primary cell walls and may in addition provoke host cell reactions by providing cell wall fragments acting as elicitors of host defense reactions (Boller and Felix 2009).

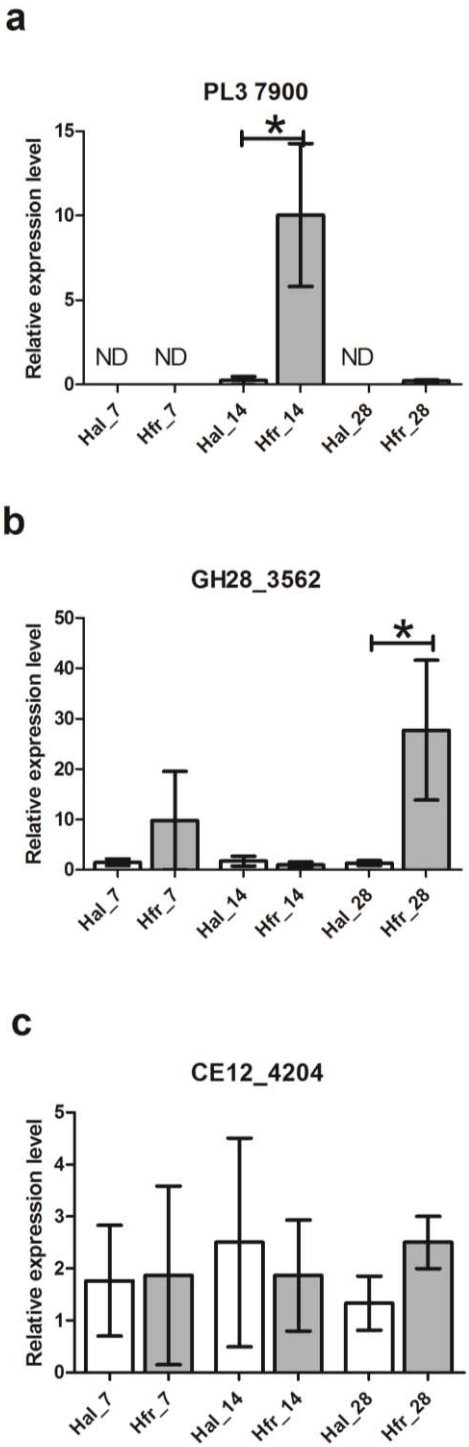

Figure 2. Expression of three *Hymenoscyphus* cell wall degrading enzymes in interactions with *Fraxinus excelsior*. Relative gene expression levels in *H. fraxineus* (shaded bars) and *H. albidus* (open bars) at 7, 14 and 28 dpi in *F. excelsior* bark: Pectin lyase PL3_7900 (A); Pectin degrading GH28_3562 (B); Cellulose degrading CE 12_4204 (C). Error bars indicate SE. Horizontal bars indicate pair-wise comparisons and asterisks indicate significances (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test)

The two *Hymenoschyphus* species both contained 17 GH18 genes, predicted to encode chitinases. GH18 genes can be divided into the defined GH18 subgroups GH18_A, GH18_B, GH18_C and ENGases (Seidl-Seiboth et al. 2014), and both *Hymenoscyphus* spp. possessed seven, three, four and two members of the respective class (Table 3). Subgroup GH18_C (killer toxin-like chitinases) was significantly expanded ($p = 0.002$) in the clade containing *Hymenoscyphus* spp. and *G. lozoyensis* compared to the other Helotialean species.

Table 3. Summary table of genome content of chitin degrading enzymes of Glucoside hydrolase family GH18 for nine Helotialean

	GH18_A	GH18_B	GH18_C	ENGase	GH18_ALL
H. albidus	7	3	4	2	17
H. fraxineus	7	3	4	2	17
G. lozoyensis	4	2	7	2	15
A. sarcoides	4	5	1	1	11
M. brunnea	5	1	0	1	7
S. borealis	7	4	5	2	18
B. cinerea	3	4	1	2	10
S. sclerotinorum	4	5	3	2	14
B. graminis	7	1	0	1	9

When examining the PCW-degrading enzyme profiles of the genomes of *H. albidus* and *H. fraxineus,* it appears that both fungi have a similar potential to degrade plant cell walls. Both species and the most closely related Helotialean fungus, the saprotrophic *G.* appear to utilize a large battery of LPMOs (AA09) to degrade cellulose (Morgenstern et al. 2014) The AA09 are oxidative enzymes that boost the enzymatic conversion of recalcitrant polysaccharides, in particular cellulose. This number of AA09 enzymes is clearly larger for *Hymenoscyphus* and *Glarea* than for other members of the Helotiales. Expansions of the LPMOs has been observed among saprotrophs, and some necrotrophs, but not among hemibiotrophs in *Dothideomycetes* (Ohm et al. 2012). This finding is an indication of the importance of saprotrophic part of the life cycle of *Hymenoscyphus* spp. Similarily, the hemicellulose degrading potential is also higher for the *Hymenoscyphus* spp. and *G. lozoyensis* than for the true necrotrophic/hemibiotrophic helotialean fungi, again suggesting an important role for the saprotrophic degradation of plant cell walls.

The interest in the LPMO enzymes stemmed from their ability to stimulate biomass hydrolysis of cellulose by giving an oxidative enzyme boost to the enzymatic conversion of recalcitrant polysaccharides. A putative additional

role for AA9 LPMOs in host invasion has also been suggested for an AA9 enzyme of the phytopathogen *Pyrenochaeta lycopersici*, following tomato root infections (Valente et al. 2011). Strong induction of *P. lycopersici* AA9 *Plegl1* transcription at 96 h after infection of tomato roots coincides with the switch from biotrophic to necrotrophic fungal growth (Goodenough et al. 1976, Valente et al. 2011). Similar importance in necrotrophic growth may be present for *H. fraxineus*.

Obviously the genomic CAZYme arsenal could be used differently by fungi. Consequently, our observation of such a pronounced similarity in genomic PCW-degrading enzyme profiles between *H. albidus* and *H. fraxineus*, and also with the saprotroph *G. lozoyensis,* does not prove that *H. albidus* and *H. fraxineus* use similar strategies in their interactions with *F. excelsior*. Instead we think that the similarity in genomic PCW-degrading enzyme profiles of *H. albidus* and *H. fraxinues* may be attributable to the prolonged phase of saprotrophic growth, decomposing leaves and rachises on the ground. The adaption to saprotrophic growth including frequent interactions with other fungi on the forest floor has further support in the expansion in the GH18 C group chitinases in *Hymenoscyphus* (Table 3). This group of enzymes has previously been interpreted to have a function similar to a killer toxin and to be involved in antagonistic interactions in saprotrophic life styles.

The predicted secretomes of H. albidus *and* H. fraxineus

Many plant-associated fungi interact with their hosts through the secretion of various proteins known as effectors, which alter host-cell structure and function. Based on SignalP and Phobious annotations done through InterPro Scan, we identified 2160 and 2006 secreted genes in *H. fraxineus* and *H. albidus*, respectively. This is similar to the number found in *G. lozoyensis*, but almost doubled as compared to the other Helotealian species**.** We further identified 308 and 294 short secreted proteins (SSPs) less than 150 amino acids in length in *H. fraxineus* and *H. albidus*, respectively. This represented more than a two-fold increase in SSP gene number as compared to *G. lozoyensis*, and a three to six-fold increase in comparison to the other Helotialean species of this study and *B. graminis*.

Genes of effector families often share common sequence motifs, such as the RXLR motif of oomycte effector proteins or the YxC effectors of *B. graminis* (Spanu et al. 2010). We mined the SSP sets of all studied species for conserved sequence motifs using MEME (Bailey et al. 2015) from the MEME package and then used FIMO to annotate all occurrences of the discovered motifs in SSPs and all other genes. We validated the approach by running it on *B. graminis* where we recovered the YxC motif, which shows a significant 10-fold increase in occurrence among the *B. graminis* SSPs (Fisher's exact test, $p < 10^{-9}$) but not overrepresented among *Hymenoscyphus* SSPs. Next, we analysed the occurrence of motifs in *Hymenoscyphus* spp. SSPs and found three motifs each in *H. fraxineus* and *H. albidus* that were significantly ($p < 0.0001$) 2-3 fold overrepresented (Figure 3). Two of the motifs, "CxDC" and "$CxSx_4I$" were shared between the species. The latter two motifs often occurred in tandem within the SSP genes where they occurred.

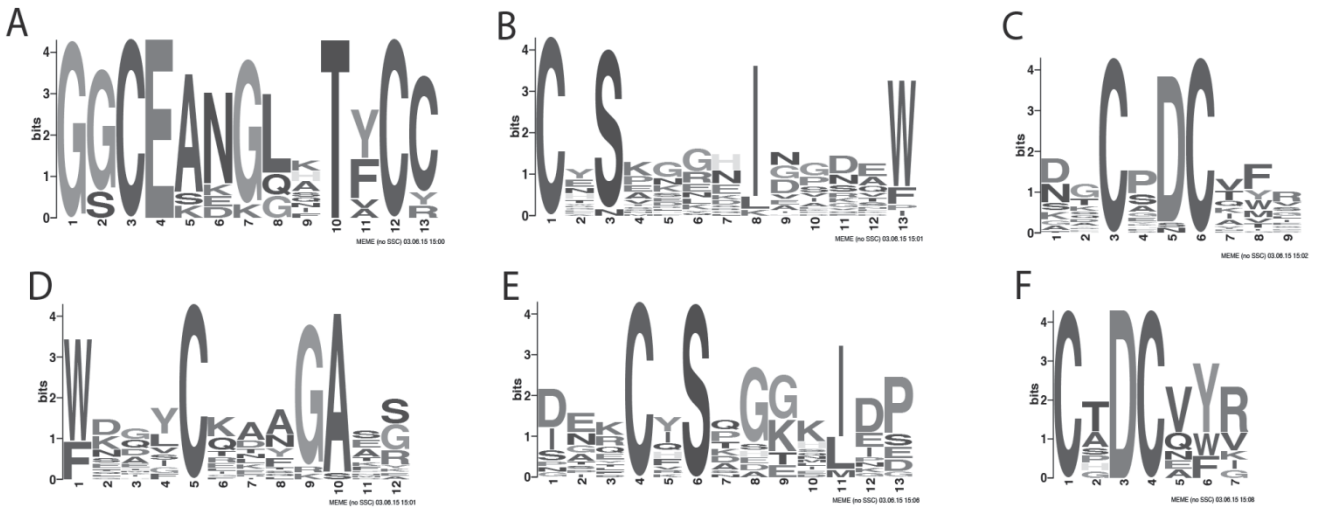

Figure 3. Overrepresented sequence motifs in *H. fraxineus* (A-C) and *H. albidus* (D-F) SSPs. The conserved sequence motifs of *H. fraxineus* and *H. albidus* occur in about 10% of the predicted SSPs (A 23, B 30 and C 33 times in *H. fraxineus.* D 31, E 29, F 27 times in *H. albidus*)

Genes encoding proteins involved in host-parasite interaction are expected to show elevated levels of evolutionary turnover. When amino acid conservation in SSPs between the two *Hymenoscyphus* species was analysed, only 158 ascertained one-to-one orthologous SSP pairs were identified, and the average dN/dS ratio for these genes was 0.6. Only 17 of these SSPs were also conserved in *G. lozoyensis*.

We mined seven available RNAseq libraries of *F. excelsior* infected with *H. fraxineus* (Eshghi Sahraei et al. unpublished), as well as a pure culture RNAseq library (generated in the current study) for expression of the predicted SSPs. Only two *H. fraxineus* SSPs were expressed, HYFRA_T00003917 (protein of unknown function) and HYFRA_T00003226 (similar to SnodProt1). Both genes were expressed *in planta* as well as in pure culture. HYFRA _T00003226 shows similarity to the cerato-platanin family of proteins known from other plant pathogens. No expression of *H. albidus* SSP genes was observed, neither *in planta* in two libraries nor in a single library of pure culture RNASeq data.

Cerato-Platanin (CP) is a non-catalytic phytotoxic hydrophobin-like small secreted protein, which work as virulence factors and/or as elicitors of defense responses and systemic resistance, thus acting as PAMPs (pathogen-associated molecular patterns). Moreover, CP has been defined an expansin-like protein showing the ability to weaken cellulose aggregates, like the canonical plant expansins do (Pazzagli 1999, Luti et al. 2016). The secreted CP-like protein of *H fraxineus* may also protect fungal cell wall polysaccharides from enzymatic degradation by the host in a manner analogous to what recently was suggested for *Fusarium graminearum* (Quarantin et al. 2016).

***Metallopeptidases genes are differentially expressed* in planta**

It has been suggested that fungal metallopeptidases, fungalysins, can target and degrade non-structural defense proteins *in planta* (Jashni et al. 2015, Naumann et al. 2011). Therefore we examined the metallopeptidase and serine protease gene families in *H. fraxineus* and *H. albidus* and found relatively few significant differences in gene family size between the two genomes or in comparison with other Helotialean fungi (Figure 4). However, it is noteworthy that *H. fraxineus* and *H. albidus* each carry two genes of the metallopeptidase families M35 (deuterolysin) and M36 (fungalysin) while the genomes of all other Helotialean fungi, except *G. lozoyensis* and *M. brunnea,* possessed no M36 genes**.** *H. fraxineus, H. albidus* and *G. lozoyensis* also contained seven protease genes from family M28A while most other Helotialean fungi only possess two. The expression of M35, M36 and two M28A genes from *H. fraxineus* and *H. albidus* were analysed with qRT-PCR at 7, 14 and 28 dpi on *F. excelsior*.

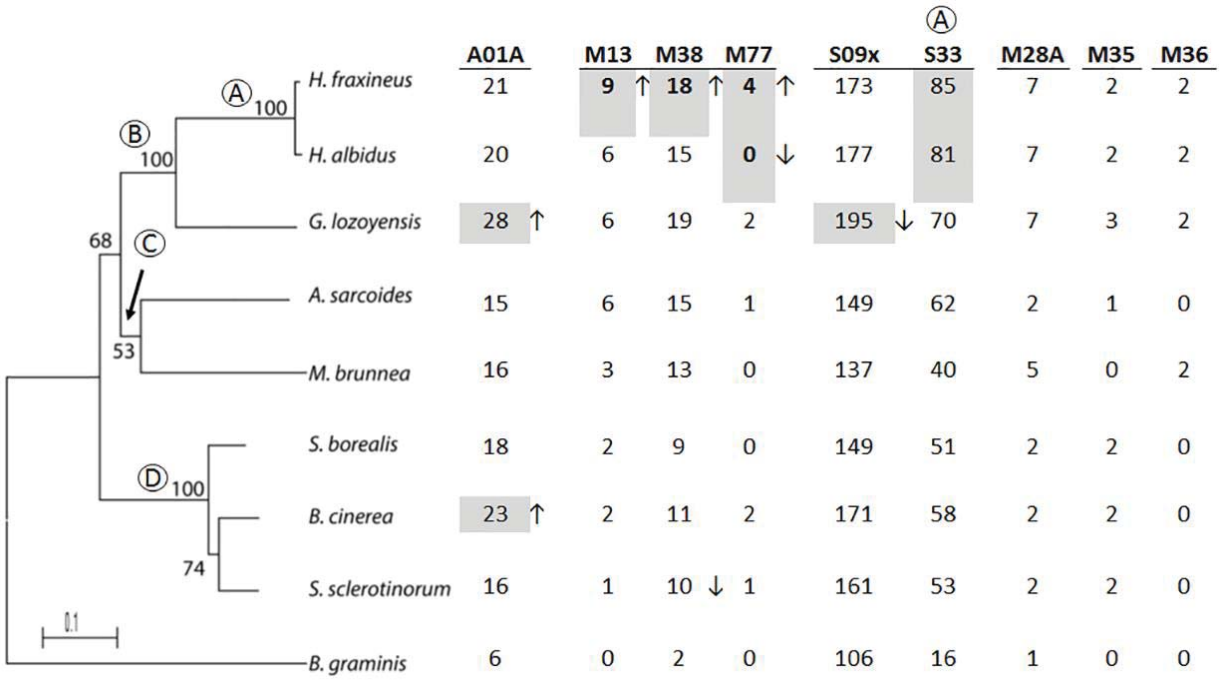

Figure 4. Expansions and contractions among proteolytic gene families in the helotiales: Gene families with significant (Viterbi *P*-value < 0.05) expansions or contractions in specific taxa or ancestral species are shaded in grey. The arrows indicate expansion (↑) or contraction (↓) for individual species along the tree. Expansion or contractions in ancestral species are indicated by encircled A, at the node in question and above the columns of gene families. Numbers in bold font indicate expansions or contractions in the *Hymenoscyphus*

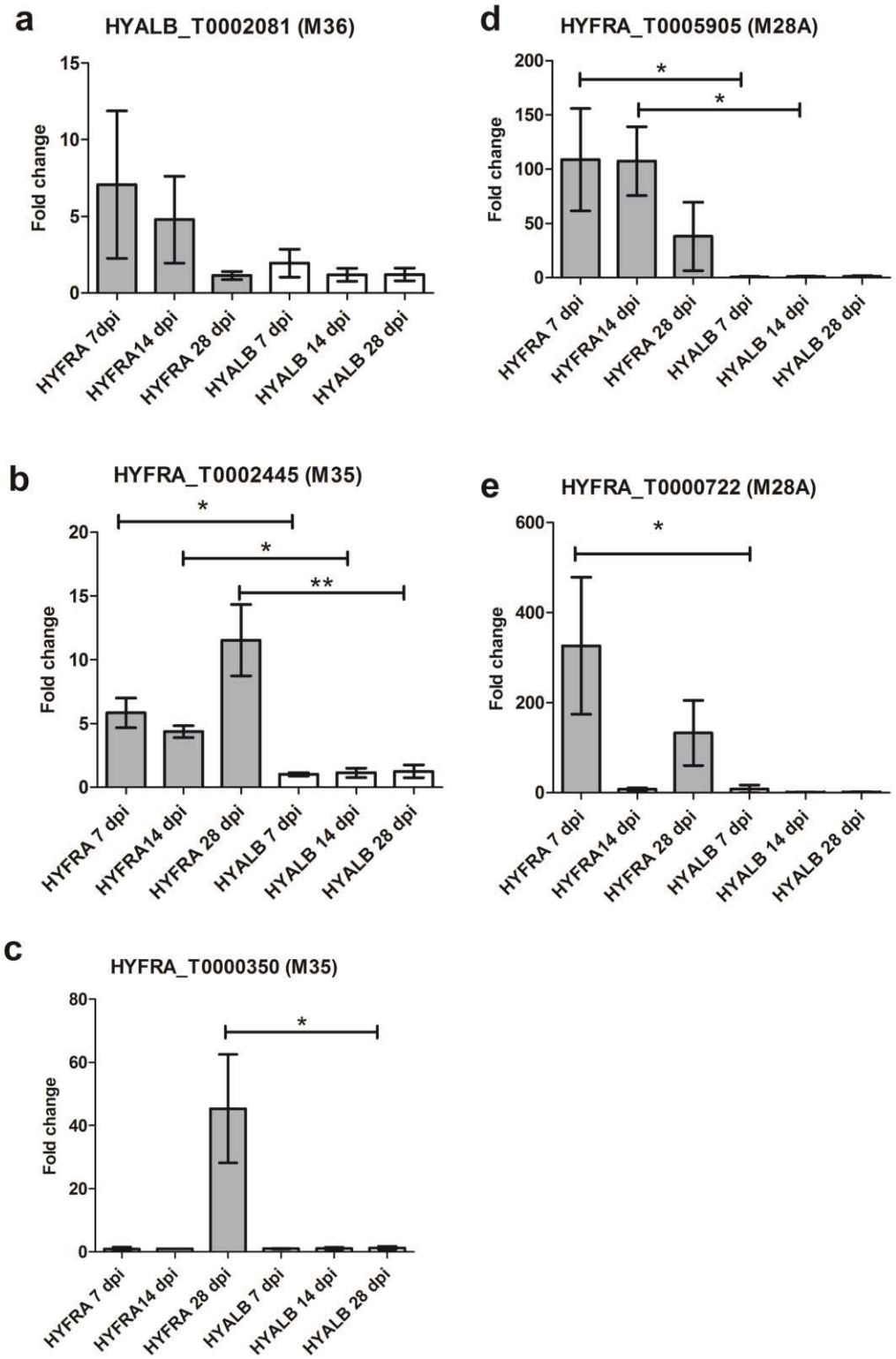

Figure 5. Expression of *Hymenoscyphus* metallopeptidases in interactions with *Fraxinus excelsior*. Relative gene expression levels in *H. fraxineus* (shaded bars) and *H. albidus* (open bars) at 7, 14 and 28 dpi in *F. excelsior* bark: M36_2081 (A); M35_2445 (B); M35_0350 (C); M28A_2445 (D) and M28A_0722 (E). Error bars indicate *SE*. Horizontal bars indicate pair-wise comparisons and asterisks indicate significances (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test)

The two *H. fraxineus* M36 family genes HYFRA_T0002081 and HYFRA_T0004626, and their *H. albidus* orthologs, showed significantly different expression patterns *in planta*. No expression of HYFRA_T0004626 could be detected in either *H. fraxineus* or *H. albidus* interactions with *F. excelsior* (data not shown). On the other hand HYFRA_T0002081 was expressed to similar levels in both *H. fraxineus* and *H. albidus* in interactions with *F. excelsior* at all time-points analysed (Figure 5a).

Both family M35 metallopeptidases (HYFRA_T0002445 and HYFRA_T0000350) showed significantly ($p < 0.05$) higher expression levels in *H. fraxineus* than in *H. albidus* at 28 dpi (Figure 5b and c). However, the expression pattern differentiated at the earlier time-points. HYFRA_T0002445 showed significantly ($p < 0.05$) higher transcript levels in *H. fraxineus* than in *H. albidus* also at 7 and 14 dpi (Figure 5b), while HYFRA_T0000350 showed similar low transcript levels in both species at these time-points (Figure 5c).

The two analysed family M28A metallopeptidases (HYFRA_T0005905 and HYFRA_T0000722) showed very high levels of expression in *H. fraxineus* but not *H. albidus* during their interaction with *F. excelsior* (Figure 5d and e). Interestingly, HYFRA_T0000722 showed the highest degree of induction in both species at 7 dpi, albeit at significantly different levels (Figure 5e).

It is possible that one or several of these metallopeptidases represent an initial step in the arms race between ash and *H. fraxineus*, e.g. by inhibiting chitin-binding domain(CBD)-containing chitinases (Jashni et al. 2015). This may lead to co-evolutionary diversification and adaptation shaping pathogen lifestyle and host defense in the newly formed pathosystem of ash dieback.

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