Genomic innovation and horizontal gene transfer shaped

plant colonization and biomass degradation strategies of a

globally prevalent fungal pathogen

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47 Abstract

Members of the fungal genus *Armillaria* are necrotrophic pathogens with efficient plant biomass-

degrading strategies. The genus includes some of the largest terrestrial organisms on Earth,

spreading underground and causing tremendous losses in diverse ecosystems. Despite their

global importance, the mechanism by which *Armillaria* evolved pathogenicity in a clade of

dominantly non-pathogenic wood-degraders (Agaricales) remains elusive. Here, using new

genomic data, we show that *Armillaria* species, in addition to widespread gene duplications and

de novo gene origins, appear to have at least 775 genes that were acquired via 101 horizontal

gene transfer (HGT) events, primarily from Ascomycota. Functional and expression data

suggest that HGT might have affected plant biomass-degrading and virulence abilities of

Armillaria, two pivotal traits in their lifestyle. We further assayed gene expression during root

and cambium colonization, and report putative virulence factors, extensive regulation of

horizontally acquired and wood-decay related genes as well as novel pathogenicity-induced

small secreted proteins (PiSSPs). Two PiSSPs induced necrosis in live plants, suggesting they

are potential virulence effectors conserved across *Armillaria*. Overall, this study details how

evolution knitted together horizontally and vertically inherited genes in complex adaptive traits,

such as plant biomass degradation and pathogenicity, paving the way for development of

infection models for one of the most influential pathogens of temperate forest ecosystems.

Introduction

Plant pathogenic fungi cause significant economic losses worldwide in a wide variety of plant species, including forest trees. Among tree pathogens, the genus *Armillaria* (Basidiomycota, Agaricales, Physalacriaceae) stands out as one of the most important in temperate systems, responsible for great losses in both natural and planted stands of woody plants [1–3]. Known at the genus-level as *Armillaria* root-rot disease [3–5], the most common pathogenic species is *A. mellea sensu lato,* which has been reported to infect >500 plant species [1,6,7] and is solely responsible for up to 40% annual loss of vinegrape in California [8]. *A. ostoyae* is responsible for significant losses in conifer forests [9,10].

   Armillaria species evolved a range of features exceptional among fungi, which have conceivably all emerged in its most recent common ancestor (MRCA). These include a very low mutation rate, extreme longevity and immense colony sizes (>2,500 years, >900 hectares [1,3,11–13]), diploidy [14], bioluminescence [15], specialized underground structures known as rhizomorphs [16,17], and the potential to fix atmospheric N_2 [18]. Perhaps the most economically important aspect of this genus is the ability to infect and kill woody plants [1,2,19,20]. Most *Armillaria* species are broad host range necrotrophs [1,3]. After infection through the roots, they colonize and kill the cambium of the tree, causing the death of the plant and enabling the fungus to transition to its necrotrophic phase [1,21]. Despite a well-documented epidemiology and etiology [2,19,21–24], molecular aspects of the infection process are poorly known. Recent studies and genome sequencing efforts highlighted certain secondary metabolites, plant cell wall degrading enzymes (PCWDEs), chitin-binding proteins and expanded protein-coding repertoires enriched in putative pathogenicity-related genes, among others [1,17,18,25–30]. It is likely that these previous findings only cover a fraction of the virulence genes of *Armillaria*, leaving much of its pathogenic arsenal yet to be characterized.

   As a result, it is unknown how infection models developed based on better-studied necrotrophic fungi (e.g. *Sclerotinia sclerotiorum* [31–33], or *Colletotrichum* spp. [34]), are applicable for *Armillaria*, if at all, and what traits the latter evolved for plant infection, are unknown. In other necrotrophs, broad roles of tissue acidification, tolerance towards and detoxification of plant secondary metabolites and reactive oxygen bursts [35–37], secretion of diverse plant cell wall degrading enzymes (PCWDEs) [34,38] and effectors [39] have been established as key assets of infection. However, *Armillaria* represents an independent origin of necrotrophy, which might have resulted in unique infection mechanisms, our understanding of which remains limited.

Here, we sequenced eight new genomes and report transcriptome data from new *in*
planta and *in vitro* pathosystems, allowing us to explore genome evolution and key aspects of
the necrotrophic lifestyle of *Armillaria* spp. We infer that gene duplications, genus-specific gene
families and horizontal gene transfers have shaped the genomic toolkit available for plant
infection and biomass degradation. RNA-Seq data from six experiments and four *Armillaria*
species, including new *in planta* time series- and fresh stem invasion experiments allowed us to
decipher gene expression patterns specific for these processes in virulent and non-virulent
strains. Experimental validation of predicted pathogenicity-induced small secreted proteins
revealed potential conserved virulence factors in *Armillaria*. Overall, our phylogenomic and gene
expression studies elucidate how vertically and horizontally acquired genes became integrated
into complex adaptive traits of a globally important fungal group.

Results

New *Armillaria* genomes

We report the high-quality annotated *de novo* genomes of eight *Armillaria* species including
A. borealis, *A. ectypa*, *A. fumosa*, *A. luteobubalina, A. mellea*, *A. nabsnona*, *A. novae-*
zealandiae and *A. tabescens.* The new genomes were assembled with Falcon [40] to 33-864
scaffolds comprising 40-79 Mbp haploid size, with 12,228 - 19,984 predicted gene models and
BUSCO (fungi) completeness 97.7-99.7% (Figure 1). Sequencing statistics for the new
genomes are summarized in Table S1.
We sampled all major clades recognized currently [41], including the Northern Hemisphere (*A.*
borealis, A. ostoyae, A. gallica, A. cepistipes, A. solidipes, A. nabsnona), the
Australasian/Southern American (*A. luteobubalina, A. fumosa, A. novae-zelandiae*), African (*A.*
fuscipes) and melleoid clades (*A. mellea*). We also included 2 species from subgenus
Desarmillaria (*A. tabescens* and *A. ectypa*), of which the moss-associated *A. ectypa* had the
smallest genome among *Armillaria* spp., as well as *Guyanagaster necrorhizus* [18], which is the
sister genus of *Armillaria* [41] (Figure 1). For comparative analyses, we annotated carbohydrate
active enzymes (CAZymes), plant cell wall degrading enzymes (PCWDEs), secreted proteins
and small secreted proteins (SSPs) (<300 amino acids).
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Figure 1: **General genome statistics and reconstruction of ancestral genome size**

dynamics for 15 *Armillaria* species and 5 Physalacriaceae outgroups. Numbers at nodes

represent ancestral proteome sizes in the Physalacriaceae tree. Purple dot corresponds to sizes

for each node in the tree (for gene gains and losses at each node, and for the complete species

tree, see Figure S1). Blue dots represent BUSCO scores for each species. For proteome sizes,

darker color shows proteins with no known functional domains (unannotated proteins).

Carbohydrate active enzymes (CAZymes) - darker color shows plant cell wall degrading

enzymes (PCWDEs), lighter color shows other CAZymes. Secretomes and SSPs - darker color

shows unannotated proteins, lighter color shows proteins with known functional domains (for

general genome statistics of all 66 species in Dataset 1, see Table S1).

Genetic innovations in *Armillaria* clade

To analyze the genomic innovations associated with the rise of *Armillaria*, we used genomes

of 15 *Armillaria* species and 5 outgroups from the Physalacriaceae, which increased sampling

density nearly four-fold compared to previous comparative studies [17,25]. We combined these with

those of other Agaricales exhibiting a range of lifestyles, resulting in a dataset of 66 species

(Table S1).

Reconstruction of genome-wide gene gain/loss patterns revealed genome expansion in

Armillaria. The estimated ancient gene set at the root node comprised 9,929 genes, suggesting

an early origin for most genes (Figure S1). The *Armillaria* genus showed a net genome

expansion with 18,662 protein-coding genes inferred for the most recent common ancestor

(MRCA) of *Armillaria* (2,913 duplications, 189 losses), as opposed to 15,938 for the MRCA of

Armillaria and *Guyanagaster* (2,158 duplications, 4,375 losses) and 18,155 for the MRCA of

Armillaria, *Guyanagaster* and *Hymenopellis* (653 duplications, 5,187 losses) (Figure 1, Figure

S1). The MRCA of the Northern Hemisphere clade was inferred to have undergone further

expansion to 23,736 genes (3,140 duplications, 1,525 losses). These data indicate that the

large protein coding repertoires of *Armillaria* spp. can be explained by genus-specific gene

duplications, as suggested before [17].

The 2,913 duplications in the MRCA of *Armillaria* happened in a total of 1,473 orthogroups

(OGs). Gene ontology (GO) and InterPro enrichment analyses for these showed significant

overrepresentation of 55 molecular functions, 18 biological processes and 3 cellular component

terms (using topGO [42], weight01 algorithm, p <0.05) (Figure S2) and 733 InterPro terms (Table

S2). These included functions related to plant biomass utilization, such as pectin-degradation

(pectate lyases, pectin lyases, pectin esterases, GH28), cellulose binding, and putative

extracellular and aromatic compound breakdown (e.g. intradiol ring-cleavage dioxygenase,

multicopper oxidases) (see Table S2, Figure S2). Duplicated genes were also enriched in

putative pathogenesis related gene families. For instance, we found significant

overrepresentation of deuterolysins, aspartic peptidases, chitin deacetylases, and SCP-like

Golgi-associated pathogenesis-related proteins. Ceratoplatanins and LysM domains, which

were reported to assist infection in pathogenic fungi [43–45] were also enriched in *Armillaria*.

Small secreted proteins (SSPs), which include cysteine-rich proteins involved in, among

others, host colonization [46], were found in 270-507 copies in *Armillaria*, with *A. fuscipes* having

the fewest and *A. luteobubalina* having the most. Of these, 45-57% had no known functional

domains, which we hereafter refer to as unannotated SSPs. Typically this latter class of SSPs

are called candidate secreted effector proteins (CSEPs) in pathogenic fungi [34,47,48].

Gene families that arose within and are conserved in most *Armillaria* spp., referred to as

novel core orthogroups, may be particularly relevant for explaining the plethora of *Armillaria*-

specific innovations. We found 212 novel core orthogroups shared by at least twelve *Armillaria*

species including *G. necrorhizus* (Table S2, Figure S3). Of these, 116 consisted of proteins with

no known functional annotations. The remaining orthogroups were dominated by F-box

domains, Leucine-rich repeats, Cytochrome P450s, Zinc-finger C2H2 type transcription factors,

protein kinases and other fast evolving gene families (Table S2).

Armillaria spp. are among the few bioluminescent fungi [15]. Ke et al. reported the luciferase

gene cluster in *Armillaria,* comprising five genes [49]. We found the luciferase cluster to be

conserved and highly syntenic in all *Armillaria* genomes (Figure S4), however, the cluster was

missing in other Physalacriaceae, suggesting it was lost in those, or gained in the common

ancestor of *Armillaria* and *Guyanagaster*.

Plant biomass degrading enzymes in *Armillaria* and the Physalacriaceae

Ecologically, *Armillaria* species are reported to be facultative necrotrophs that first kill the

host, then utilize its biomass during the saprotrophic phase. The new genomes allowed us to

make predictions on the wood decay strategy of *Armillaria*, relative to other wood-decaying fungi

based on their plant cell wall degrading enzyme genes (PCWDEs). Similar to white rot (WR)

fungi and necrotrophs [39,50], *Armillaria* species possess the complete enzymatic repertoire for

degrading woody plant biomass (Figure 1). We generated phylogenetic PCAs for *Armillaria*

species, other Physalacriaceae, WR and litter decomposer (LD) species (Table S1) based on

PCWDEs acting on cellulose, hemicellulose, pectin and lignin. Cellulases and pectinases clearly

separated *Armillaria* spp. and other Physalacriaceae from WR and LD fungi (Figure 2, Figure

S5), whereas hemicellulase and ligninase PCA-s grouped them together (Figure 2, Figure S6,

Figure S7, Figure S8). Cellulase loading factors indicate that this separation was mainly driven

by expansins, the AA16, AA8, and AA3_1, GH1 and GH45 families. In line with loading factors,

Armillaria and other Physalacriaceae have more AA3_1, GH1 and GH45 genes than WR and

LD fungi (Figure S9). On the other hand, they were depleted in CBM1 and GH5_5 with an

average of 12 and 5 genes respectively, compared to WR and LD genomes encoding 20-60

CBM1 and 4-10 GH5_5 genes. The GH44 family, which is specific to Basidiomycota [51], is

missing in the Physalacriaceae and *Armillaria*, indicating that gene losses drove trophic mode

evolution of these fungi towards an Ascomycota-like lifestyle.

In the pectinase PCA, the highest loading family was CBM67 (rhamnose binding modules),

which were enriched in *Armillaria* species and were absent in most WR species in our dataset

(Figure S10). CBM67s are frequently associated with GH78 and PL1 proteins [52]. The PL1 family

is present in all Physalacriaceae species, but is depleted or missing in many WR and LD fungi

(Figure S10). Other pectin-acting families, such as GH28, GH53, GH88, CE8, and CE12, were

present in higher numbers in *Armillaria* and other Physalacriaceae than in WR and LD fungi,

whereas PL1_7, PL26, PL3_2, and PL4_1 were abundant in *Armillaria* species but absent in

most WR and some LD species.

These analyses portray *Armillaria* and the Physalacriaceae as versatile wood decayers that

are nevertheless distinct from WR, despite previous classifications as such [3,17,26,53,54]. This is

consistent with microscopy, chemical and transcriptomic analyses [54–59], which indicated that

their decay chemistry is similar to soft rot [55,59], which is known only in the Ascomycota. These

observations prompted us to systematically look for similarities between PCWDE repertoires of

the Physalacriaceae and the Ascomycota. We found 16 CAZy orthogroups that were

significantly overrepresented in both groups with respect to WR+LD fungi (BH-corrected p-value

>0.05, Fisher's exact test (Table S3, Figure 2). These included several of the high-loading

families from the PCAs, as well as other PCWDEs acting on cellulose (AA3_1, AA8, CBM1),

pectin (PL3_2, PL1_7, PL9_3), cellulose/chitin (AA16), and hemicellulose (GH31, GH43, GH93,

CE4). These families could either be the result of co-expansion in both the Ascomycota and the

Physalacriaceae or represent horizontal gene transfer (HGT) events. Blast searches with

Armillaria GH28 genes suggested the latter scenario to be more likely, which led us to

systematically evaluate the role of HGTs.

Figure 2: **Plant biomass degradation genes in *Armillaria*.** A) Phylogenetic PCAs for PCWDE

gene families. Species abbreviations are shown only for Physalacriaceae species (all species

names and PCA loadings are given in Figure S5 and Table S3). B) Boxplot of copy numbers of

16 CAZy orthogroups co-enriched in Physalacriaceae and in Ascomycota with respect to WR

and LD fungi. Scale limits for the boxplot were set to 14, losing one sample point (Conioc1 in

OG0000781 with 18 genes). Lifestyles of the species used are denoted by colors for both A and
B.

Widespread horizontal transfer of genes from Ascomycota

To identify horizontally transferred (HT) gene candidates, we calculated the Alien index (AI)
[60] for each *Armillaria* and Physalacriaceae protein based on a phylogenetically broad set of 942
species from the Ascomycota, Mucoromycota, early-diverging fungi as well as plants and
bacteria (Table S1, Dataset3). Based on AI>1, we identified 99-195 HT gene candidates per
species, with *A. gallica* having the most and *A. ectypa* the fewest in *Armillaria*. Among
Physalacriaceae, *H. radicata* had the highest estimated number of HT genes (247), and *F.*
velutipes the lowest (88) (Figure 3A , Table S4).
Based on the top putative donor species from the AI data, we assembled a dataset
comprising the 20 Physalacriaceae, 90 Basidiomycota, 210 Ascomycota, 7 Mucoromycota, and
2 Zoopagomycota species. Using OrthoFinder (v.2.5.4), we identified 649 orthogroups
containing at least one gene predicted as HT by AI. Due to the similarity of the CAZy repertoires
of Physalacriaceae to Ascomycota (see above), we also included all CAZy orthogroups in these
analyses. In total, we analyzed 675 orthogroups by combining phylogenetic support (at least
70% bootstrap in ML gene trees), and top hits against the UniRef100 database [61]. Overall we
recovered 101 strongly supported horizontal transfer events (Figure 3 A) into Physalacriaceae,
corresponding to 1089 individual genes. These events were associated with 85 orthogroups,
consistent with estimates based on the Alien Index, and an additional 3 CAZy orthogroups,
suggesting AI to be an effective strategy for screening HGT candidates. Multiple internal nodes
of the Physalacriaceae tree were identified as putative recipients, most of which were deep in
the family. 88 HGT events were confidently associated with Ascomycota as donors, in particular
the Sordariomycetes and Dothideomycetes (28 transfers each). We find that ~45% of HT genes
have undergone one or multiple rounds of duplication (total 488 duplicated genes) within the
Physalacriaceae, indicating that HT genes were likely integrated into the life history of
Physalacriaceae and *Armillaria* therein. Expression levels of HT genes support their functionality
(Figure S11/Figure 3 C).
Among proteins descended from phylogenetically validated HT events, we found 164
CAZymes (Table S4), of which 117 belonged to families that were co-enriched in Ascomycota
and Physalacriaceae compared to WR and LD species (AA3_1, AA8, GH43, GH93, GT1,

PL3_2 and PL9_3). This indicates that the co-enrichment signal detected using the simple copy-
number based analyses above was likely created by HGT events. In addition to CAZymes, HT
genes included intradiol ring-cleavage dioxygenases, CAP domain proteins, Pyr1-like SCP
domains, as well as gene families with broad functional roles such as cytochrome P450,
peptidases, transporters and transcription factors (Table S4).

272   Taken together, we find that horizontal transfer affected several families associated with
wood-decay (e.g. AA3_1, GH43, PL3_2), as well as plant-fungal interactions (e.g. CAP domain
proteins, peptidases), which suggests that it might have shaped the plant biomass degrading
and pathogenic abilities of the genus. Given that Physalacriaceae species have been reported
to cause soft-rot-like decay on wood [54–56,58,59] and that soft-rot is classically restricted to the
Ascomycota [62], we hypothesize that HGT contributed to the evolution of plant biomass
degrading ability of these species. We speculate that the large number of putative HGT events
from Sordario- and Dothideomycetes might stem from the extensive contact of *Armillaria* ssp.
with other plant pathogens in these classes and/or their peculiar niche and longevity. Their
rhizomorphs can reach several meters in length and can contact numerous other soil microbes,
perhaps providing time windows for gene exchange. Additionally, other idiosyncrasies of
Armillaria, such as diploidy or the - as yet uncovered - mechanisms that ensure a low mutation
rate [12], could also be factors in the successful incorporation of foreign DNA in their genomes.

Figure 3. Horizontal gene transfer into *Armillaria* and the Physalacriaceae family. A)

Barplot showing the number of HT genes identified in each genome using Alien Index

calculations (yellow) and by phylogenetic validation (purple). B) Schematic representation of

donor and recipient relationships for HT events after phylogenetic validation. Size of the arrows

are proportional to the number of transfer events inferred from all the nodes belonging to a

specific donor group. The number of events for each donor group are listed along the arrows. C)

Violin plot showing expression dynamics of phylogenetically validated HT genes and vertically

transferred (VT) genes. X-axis shows the sample names and y-axis shows the log$_2$ transformed

gene expression (For expression dynamics of HT and VT genes in developmental dataset, see

Figure S11).

Armillaria spp. display distinct expression profiles for plant infection, necrotrophy and wood decay

During infection, *Armillaria* enters the host through the root system, colonizes and kills cambium cells, which leads to death of the plant and the onset of the necrotrophic phase [1]. Molecular aspects associated to this process are hardly known, with most information available on the wood-decaying phase [17,58]. To obtain a molecular perspective on these strategies and better understand how *Armillaria* spp. utilize expanding, novel core and HT genes, PCWDEs and gene groups frequently associated with pathogenicity, we produced new RNA-Seq data for two *in vitro* pathosystems (for *A. ostoyae, A. borealis and A. luteobubalina*) and re-analyzed data from 2 published studies [17,58]. New data were generated for an *in planta* time-series experiment of *A. luteobubalina* infecting *Eucalyptus grandis* seedlings from 24hrs to 2 weeks (Figure S12 A) and *in vitro* fresh stem invasion assays with highly and less-virulent isolates of both *A. ostoyae* and *A. borealis.* The latter experiment emulated the cambium-killing phase of the fungus (Figure S12 B). Published studies covered wood-decay in *A. ostoyae* and *A. cepistipes* [58] as well as rhizomorph and fruiting body development in *A. ostoyae* [17].

To obtain a species-independent picture, we aggregated differential gene expression data across experiments and calculated DEG enrichment ratios in each of 24 gene groups we defined (see Methods). These enrichment ratios reflect the proportion of differentially expressed genes in a given gene group in a given experiment and are shown as a heatmap on Figure 4 (see Figure S13 for fruiting body data). *In planta* infection, wood-decay and stem colonization as well as fruiting body/rhizomorph development showed distinct enrichment patterns. Cellulose-, hemicellulose-, pectin- and lignin-related PCWDE genes showed a clear enrichment in stem invasion and wood-decay experiments, as expected based on known functions of these genes [17,54]. Among genes upregulated on fresh stems, pectinases were most dominant, which possibly enables the fungus to spread between the bark and the sapwood. At the same time PCWDEs were depleted in the *in planta* time series experiment, indicating that *A. luteobubalina* did not induce these genes during the infection phase. This is consistent with most necrotrophic fungi expressing a limited set of PCWDEs for plant penetration and a larger battery of enzymes during the necrotrophic phase [34,38]. Other virulence-related genes include cerato-platanins [43,63], which showed an enriched upregulation at 24h and 48h in *A. luteobubalina* and in stem invasion by *A. borealis* (but not *A. ostoyae*). HT genes, including intradiol ring-cleavage dioxygenases, PCWDEs, peptidases and transporters, showed an enrichment in wood-decay and stem-

invasion experiments. Bioluminescence genes were enriched in root-invading mycelium and
rhizomorph (*A. ostoyae*) samples. In the developmental dataset SSPs, expanded, novel core
and stress-related genes were found to be enriched in various developmental stages of the
fungus (Figure S13). Genes related to oxidative stress were upregulated in various stages of *in*
planta infection and in stem invasion assays (Figure 4, Figure S14). Superoxide dismutases,
catalases and members of the glutathione system and ergothioneine pathway were enriched
among upregulated genes in the *in planta* invasion assays, whereas glutathione-S-transferases
and catalases were enriched in wood-decay related experiments.

It is also evident from Figure 4, that there are species- and strain-specific enrichment
patterns among up- and downregulated genes. For example, the virulent *A. borealis* A6 strain
had a unique enrichment of pathogenicity-related LysM proteins and cerato-platanins, which
was not seen in the less-virulent A4 strain or in *A. ostoyae*. Nevertheless, in both *A. borealis*
and *A. ostoyae*, the virulent strains (A6 and C18, respectively) showed more enrichment across
most gene categories tested. The species- and strain-specific enrichment patterns also suggest
that each species mount a different response under the examined conditions, which might
correlate with differences in lifestyle or virulence.

Figure 4: **Enrichment of differentially expressed genes of wood-decay, pathogenicity,**
stress-response and other gene families in 6 RNA-Seq datasets. The heatmap shows
enrichment ratios for 24 gene groups (x-axis) from aggregated differential gene expression data
across 6 experiments (A - upregulated, B - downregulated genes). Y-axis shows the sample

comparison for each dataset, with number of DEGs shown as a barplot at right. In the hatmaps,
warmer colors mean higher enrichment ratios (for a complete list of odds ratios Table S5).

Early stages of live host colonization by *Armillaria* characterized by complex transcriptomic regulation

To gain a mechanistic understanding of early stages of *Armillaria* infection, we analyzed
gene expression of *A. luteobubalina* colonizing *E. grandis* roots across five time points (pre-
symbiosis, 24hrs, 48hrs, 1 week and 2 weeks) in four biological replicates and compared these
to non-symbiotic samples (Figure S15, Table S5). SuperSeq revealed that the analysis detected
77-94% of differentially expressed genes (DEGs) in *A. luteobubalina* across the different
timepoints, whereas, in *E. grandis*, we could variably detect 25-70% of DEGs (Figure S16).
These genes were arranged into modules based on expression similarity using the short time-
series expression miner (STEM) [64]. This yielded eight and four significant modules in the fungus
and plant, respectively. Five fungal modules showed gradual increase in expression during time,
we refer to these as pathogenicity-specific profiles (Figure S17). Genes in these were enriched
for GO terms often associated with pathogenic interactions [65–67], with the strongest signal for
(oxidative) stress (Figure S17, Figure S14). Inspection of *A. luteobubalina* DEGs identified
genes that relate to four pathogenic processes: host immune suppression, oxidative stress,
detoxification, and cytotoxicity (e.g. cerato-platanins) [68]. Glutathione-S-transferases were almost
uniformly upregulated in plant-associated samples, compared to free-living mycelium, whereas
other genes related to oxidative stress response (e.g. superoxide dismutases) were mostly
upregulated at 1 and 2 weeks (Figure S14). Toxin efflux systems, which are involved in the
tolerance against plant secondary and defense metabolites (e.g. phytoalexins, phytoanticipins),
have been reported to be transcriptionally regulated in necrotrophs [37]. Detoxification of plant
antifungal isoflavones by *A. mellea* has been described [69], although the cellular pathways
involved are unknown. Membrane transporters, cytochrome p450 monooxygenases and
laccases, which are often associated with detoxification or plant metabolites [37,70], showed
extensive regulation during the time course of the experiment, with clusters of upregulated
genes at 1 and 2 weeks (Figure S14). On the other hand, we did not observe extensive
regulation of PCWDEs, which is consistent with patterns reported for data on early colonization
of other fungal pathogens (Figure 4).
We observed an upregulation of HT genes at certain stages of *in planta* colonization. Of the
61 phylogenetically validated HT genes in *A. luteobubalina*, 23 were differentially expressed at

some time point, however, without any significant enrichment at any individual time point (Figure

4, Table S5). We identified two horizontally acquired CAP domain/pathogenicity-related-1 (PR-

1) proteins which were strongly upregulated across the time-series and three LysM domain

proteins (CBM50) that were upregulated at 24 and 48 hours. Both PR-1 and LysM genes are

known to be involved in fungal pathogenicity, by facilitating the transport of fatty acids and

sterols [71,72] and masking the presence of chitin residues from the plant immune system,

respectively [73,74].

When compared to *A. luteobubalina,* DEGs of its host, *E. grandis,* were found to assemble

into four significant expression modules. These were enriched in defense-related terms,

including chitinase activity, chitin binding, defense response to fungus, defense response to

bacterium (Figure S18), suggesting an activation of plant immunity during the early stages of

fungal colonization. A finer level analysis of the plant response identified that very few plant

genes were differentially regulated across the whole time course, with 95 DEGs up-regulated

across the whole time course and 59 DEGs repressed in the same series (Figure S19). Within

the core up-regulated genes we found enrichment for phosphoprotein phosphatase activity due

to up-regulation of PP2 family genes related to effector immunity and ABA-JA cross-talk [75].

Other GO terms enriched in this gene set included unfolded protein binding/chaperone activity,

induction of heat shock proteins, and jasmonic acid response due to PR4 and MYB108 gene

induction [76,77]. Of the core repressed genes, we found GO enrichment for copper ion binding

proteins, peroxidase activity, carboxylic acid binding, hydrolase activity, and lipid transfer.

Reduction of copper ion binding proteins, peroxidase activity and lipid transfer would, likely, lead

to a delayed hypersensitive response as copper ions are necessary for ethylene production and

repression of the ABA biosynthesis [78] while peroxide activity directly leads to plant cell death

and the lipid-associated genes encode DIR-like proteins responsible for long-range immune

signaling [79]. The genes associated to carboxylic acid binding and hydrolase activity, meanwhile,

likely affect pathogen nutrition more directly as the repressed genes associated to the former

are involved in biosynthesis of L-serine, lack of which would reduce the nutrition value of *E.*

grandis tissues, while the latter are involved in detoxification (i.e. cyanoalanine nitrilase) which

could lead to a toxic build-up of 3-cyano-L-alanine thereby inhibiting pathogen growth [80].

Putative necrotrophic SSPs identified and tested experimentally

Phytopathogenic fungi often utilize effectors that suppress or manipulate host defense

responses [39,81,82]. As many effectors are SSPs, we scrutinized the 39 and 38 annotated and

unannotated SSPs, respectively, of *A. luteobubalina* that were upregulated in at least one stage

during the time series (Figure 5 and Figure S20). Annotated SSPs with highest fold changes

(hereafter FC) included Pry1-like, SCP- or CAP-, Ricin-B-like lectin- and LysM domain

containing genes (Table S6), which are related to fungal virulence. Two unannotated SSPs

(1165297 and 1348401) had the highest FC values among all genes, so we selected these for

experimental validation and call them pathogenicity-induced SSPs (PiSSPs, Figure 5 A, red

arrows). Gene 1348401 had a peak expression at 48hrs (FC = 232) whereas 1165297 at 1

week (FC = 447). The encoded proteins contain no conserved domain and have no predicted

function. Orthology searches revealed that the two PiSSPs were surprisingly conserved (Figure

5, (Figure S21, Figure S22). Homologs of 1348401 are present throughout the Agaricales and

the family strongly expanded in *Armillaria*, with on average 7 genes per species compared to

1.8 in other Agaricales (Figure S21). The non-pathogenic *A. ectypa* had the fewest genes (3) in

this family. We cloned both 1165297 and 1348401 and expressed them transiently in two plant

systems: the non-host *Nicotiana benthamiana* and the host plant *E. grandis.* In the former case,

expression of 1165297 led to a mild yellowing around the injection site, indicating a

hypersensitive response, while 1348401 did not affect the leaf health (Figure 5 B). The

expression of these two proteins in *E. grandis* leaves*,* however, led to rapid cell death within 1

week of agroinfiltration (Figure 5 B), indicating a specific interaction with plant tissue. These

data indicate that certain proteins of *A. luteobubalina* can cause necrotic lesions in host plant

tissue. The evolutionary conservation of these two proteins outside *Armillaria* is somewhat

surprising and is in contrast with observations on other (mostly Ascomycota) effectors [46,47].

Whether they trigger cell death via direct (as true effectors) or indirect routes remains to be

established. These data raise the possibility that *Armillaria* use specific mechanisms for

necrotrophy, as postulated by the gene-for-gene model [39].

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Figure 5: **Pathogenicity-induced SSP (PiSSPs) expression and conservation in *A.*
luteobubalina.** A) Heatmap shows log_2 fold changes for SSPs, upregulated in at least one time
point. Red shows higher and blue depicts lower logFC, followed by presence/absence matrix of
homologs of unannotated SSPs in 131 species (Dataset 2). X-axis shows Protein IDs for both
heatmap and presence/absence matrix. The two experimentally validated PiSSPs are shown by
red arrows. Y-axis of heatmap shows sample comparisons; and species order in
presence/absence matrix. B) Transient transformation of the non-host *N. benthamiana* and the
host *E. grandis* with either an empty vector, or with an *in planta* expression vector encoding
1165297 or *1348401.*

Potential virulence factors in *Armillaria* spp.

In vitro invasion assays using freshly cut conifer stems were set up to explore gene expression in highly and less-virulent isolates [23,24] of the conifer-specific *A. ostoyae* and *A. borealis* (Figure S12). These experiments emulated cambium colonization and killing, and virulent strains consistently colonized the stems faster. We performed differential gene expression analyses between control and invasive mycelia as well as between virulent and less-virulent strains of the same species (Table S5). SuperSeq [83] indicated that the data allowed the detection of up to 89.4-98.6% of all DEGs (Figure S16).

To uncover cambium colonization strategies shared by all strains, we scrutinized genes upregulated in invasive mycelia, relative to control mycelia (Supplementary Note 1). We identified upregulation of PCWDEs, in particular pectinases, as a major transcriptional response to fresh stems in both species. Enrichment ratios confirmed that pectin-related genes are induced in the largest proportions, followed by hemicellulases, cellulases and lignin-related genes (Figure 4). Horizontally transferred intradiol ring-cleavage dioxygenases were also upregulated in these experiments.

We also aimed to identify genes encoding potential virulence factors by comparing highly and less-virulent isolates (Supplementary Note 1). Based on manual curation and sequence-based comparison of upregulated genes against PHI-base [84], we identified 303 and 56 candidate virulence factor genes from *A. borealis* and *A. ostoyae* (Table S6). We further determined the secreted proteins to predict potential plant interacting factors. From the *A. borealis* data, 40 secreted proteins, including 9 SSPs, 5 CAZymes, 2 peptidases, 2 intradiol ring-cleavage dioxygenases and one hydrophobin-related gene could be identified. In *A. ostoyae* we found 18 genes, including 3 SSPs, 5 CAZymes and 1-1 genes representing hydrophobins, intradiol ring-cleavage dioxygenases and tyrosinases (Table S6). Notably, highly virulent strains of both species upregulated a pair of polygalacturonase (GH28) and cellulase (GH3) genes, which were both homologous to virulence effectors reported from Ascomycota [85–87] (Table S6).

Discussion

The genus *Armillaria* is a globally important group of primarily tree pathogens that evolved a range of unique traits, from extreme colony sizes to bioluminescence and represent an independent origin of broad host-range necrotrophy. In search of the genetic underpinnings of

the unique biology of these fungi, in this study we combined new genomes with transcriptomic

profiling of *in planta* and *in vitro* pathosystems. Comparative genomic analyses support the view

that *Armillaria* spp. have an expanded protein-coding repertoire and possess a complete set of

CAZymes for degrading lignocellulosic plant biomass (including lignin) [3,17,26,53,88]. However, we

also detected a considerable influx of Ascomycete genes through HGT which, based on the

expression data, seem to have influenced both the plant biomass degrading ability and

pathogenic attributes of *Armillaria*. Previous studies showed that wood-decay by *Armillaria* is

reminiscent of soft-rot [55,56,58,59], a lifestyle known only in Ascomycota. Congruently, our

phylogenetic PCA analyses separated *Armillaria* from white rot species, which is paradoxical

given that white rot is the dominant wood-decay strategy in the Agaricales [89]. Alien CAZyme

genes acquired through HGT from Ascomycota may explain this controversy and establish HGT

as a source of novelty in the evolution of fungal plant biomass degrading systems in the

Basidiomycota.

Armillaria are necrotrophic pathogens that first kill, then feed on their hosts [1,17]. Our data

provided the first insights into the molecular mechanisms of early- and late stages of

colonization of host plants. We detected genes related to four major necrotrophic processes:

host immune evasion and suppression, oxidative stress, detoxification, and cytotoxicity, as well

as specific regulation of horizontally transferred genes. The presence of pathogenicity-induced

small-secreted proteins and the lack of broad plant biomass degrading CAZyme upregulation

during early colonization supports the emerging view that, instead of a non-specific, brute-force

attack with a broad battery of CAZymes, necrotrophs use specific effectors for modulating the

plant immune system [39,90]. The expression patterns of the two PiSSPs we described are

consistent with the gene-for-gene theory of pathogenicity, which has traditionally been

discussed for (hemi-)biotrophs [91], but may apply to necrotrophs as well [39]. Determining whether

the two PiSSPs are truly specific effectors will require uncovering the mechanisms of their

action, but these data provide an entry point into investigating fine details of *Armillaria* - plant

interactions.

In contrast to early-stage infection, cambium colonization was characterized by a broad

upregulation of plant cell wall degrading CAZymes. Comparisons of low and highly-virulent

strains in these assays further provided insights into genes associated with virulence, such as

certain polygalacturonase- or cellulase-encoding genes.

Overall, this study revealed the molecular underpinnings of the lifestyle of a group of

widespread pathogens of woody plants, which, combined with existing and emerging

experimental tools [21,92,93] will facilitate research on the molecular mechanisms of plant

colonization. We propose that genome evolution in *Armillaria* relied on combined effects of multiple types of genetic innovation, including HGT, and that genes gained early during the evolution of the genus were integrated into cellular regulatory networks of plant biomass degradation and pathogenicity.

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Methods

Strains for Sequencing, Assembly and Annotation

Armillaria strains (Table S1) were inoculated on Malt Extract Agar (MEA) and incubated at 25°C

in the dark for 7-10 days. Identity of each strain was confirmed by amplifying and sequencing

the fungal internal transcribed spacer (ITS1-5,8S-ITS2) region and bacterial contamination was

checked using universal 16S primers. Strains were inoculated in Malt Extract Broth in 500ml

Erlenmeyer flasks and incubated at 25°C in the dark for 4-5 weeks until a substantial quantity of

fungal biomass was grown. Fungal mass was stored at -80°C until DNA and RNA extraction.

Prior to nucleic acid extraction, fungal tissues were homogenized using liquid nitrogen in mortar

and pestle. DNA extraction was performed using the Blood & Cell Culture DNA Maxi Kit (Qiagen

Inc.) and RNA extraction was performed using the RNeasy Midi Kit (Qiagen Inc.) as per

manufacturer's instructions. Using the DNA we again confirmed strain identity by sequencing

the ITS region and nucleic acid quantity was measured using Qubit (ThermoFisher) according to

the manufacturer's instructions.

Genomic and transcriptomic library preparation, sequencing, assembly and annotation

For *A. nabsnona*, *A. mellea*, and *A. ectypa*, 5 ug of genomic DNA was sheared to approximately

15-20 kb using Megaruptor3 (Diagenode). The sheared DNA was treated with DNA Prep to

remove single-stranded ends, and DNA damage repair mix followed by end repair, A-tail and

ligation of PacBio overhang adapters using SMRTbell Express Template Prep 2.0 Kit (Pacific

Biosciences). The final libraries were size selected with BluePippin (Sage Science) at 10 kb

cutoff size. For *A. borealis, A. fumosa, A. novae-zelandiae*, and *A. tabescens*, 5 ug of genomic

DNA was left unsheared due to marginal HMW DNA quality. The unsheared gDNA was treated

with exonuclease to remove single-stranded ends and DNA damage repair mix followed by end

repair and ligation of blunt adapters using SMRTbell Template Prep Kit 1.0 (Pacific

Biosciences). All libraries were purified with AMPure PB beads.

The PacBio Sequencing primers were then annealed to the SMRTbell template libraries and

sequencing polymerase was bound to them using Sequel Binding kit 2.0 (*A. nabsnona*, *A.*

mellea, *A. tabescens*, and *A. ectypa*), 2.1 (*A. borealis*), or 3.0 (*A. fumosa* and *A. novae-*

zelandiae). The prepared SMRTbell template libraries were then sequenced on a Pacific

Biosciences' Sequel sequencer using v3 sequencing primer, 1M v2 (*A. borealis*, *A. nabsnona*,

A. mellea, *A. tabescens*, and *A. ectypa*) or v3 (*A. fumosa*, and *A. novae-zelandiae*) SMRT cells,

and Version 2.1 (*A. borealis*, *A. nabsnona*, *A. mellea*, *A. tabescens*, and *A. ectypa*) or 3.0 (*A.*

fumosa and *A. novae-zelandiae*) sequencing chemistry with 1x360 & 1x600 sequencing movie

run times.

Filtered subread data was processed to remove artifacts and assembled together with

Falcon (https://github.com/PacificBiosciences/FALCON) version 1.8.8 (*A. ectypa*, *A. nabsnona*,

A. tabescens, and *A. borealis*) or version pb-assembly 0.0.2, falcon-kit 1.2.3, pypeflow 2.1.0 (*A.*

mellea, *A. novae-zelandiae*, and *A. fumosa*) to generate an initial assembly. Mitochondria was

assembled separately from the Falcon pre-assembled reads (preads) using an in-house tool

(assemblemito.sh (*A. ectypa*, *A. nabsnona*, *A. tabescens*, *A. borealis*, and *A. mellea*) or

assemblemito.py (*A. fumosa* and *A. novae-zelandiae*)), used to filter the preads, and polished

with Arrow version SMRTLink v5.0.1.9578 (*A. ectypa* and *A. nabsnona*), v5.1.0.26412 (*A.*

tabescens and *A. borealis*), v6.0.0.47841 (*A. mellea*) or v7.0.1.66975 (*A. novae-zelandiae* and

A. fumosa) (https://github.com/PacificBiosciences/GenomicConsensus). A secondary Falcon

assembly was generated using the mitochondria-filtered preads, improved with finisherSC [94]

version 2.0 (*A. ectypa* and *A. nabsnona*) or 2.1 (*A. fumosa*), and polished with Arrow version

SMRTLink v5.0.1.9578 (*A. ectypa* and *A. nabsnona*), v5.1.0.26412 (*A. tabescens* and *A.*

borealis), v6.0.0.47841 (*A. mellea*), or v7.0.1.66975 (*A. novae-zelandiae* and *A. fumosa*).

Contigs less than 1000 bp were excluded.

Stranded cDNA libraries were generated using the Illumina Truseq Stranded mRNA

Library Prep kit. mRNA was purified from 200 ng (*A. nabsnona*) or 1ug (all other species) of

total RNA using magnetic beads containing poly-T oligos. For *A. nabsnona*, mRNA was

fragmented using divalent cations and high temperature. The fragmented RNA was reverse

transcribed using random hexamers and SSII (Invitrogen) followed by second strand synthesis.

For all other species, mRNA was fragmented and reverse transcribed using random hexamers

and SSII (Invitrogen) followed by second strand synthesis. The fragmented cDNA was treated

with end-pair, A-tailing, adapter ligation, and 10 (*A. nabsnona*) or 8 (all other species) cycles of

PCR.

All prepared libraries were quantified using KAPA Biosystems' next-generation

sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument.

Sequencing was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent

kits, S4 flowcell (*A. fumosa*, *A. novae-zelandiae*, and *A. mellea*) or on the Illumina HiSeq2500

sequencer using TruSeq paired-end cluster kits v4 and TruSeq SBS sequencing kits v4 (*A.*

nabsnona, *A. borealis*, *A. tabescens* and *A. ectypa*), following a 2x150 indexed run recipe.

Raw reads were evaluated with BBDuk (https://sourceforge.net/projects/bbmap/) for

artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifacts were

trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads, and reads containing any

Ns were removed. Quality trimming was performed using the phred trimming method set at Q6.

Following trimming, reads under the length threshold were removed (minimum length 25 bases

or 1/3 of the original read length - whichever is longer). Filtered reads were assembled into

consensus sequences using Trinity (ver. 2.3.2) [95] with the --normalize_reads and --jaccard_clip

options.

Genomes were annotated with the support of their corresponding transcriptomes using

the JGI Annotation Pipeline. Assemblies and annotations are available from the JGI Genome

Portal MycoCosm (https://mycocosm.jgi.doe.gov) [96,97].

904 Taxon sampling and Functional Annotations

We used 3 datasets in this study. Dataset1 comprises 66 species (64 Agaricales, 2

Boletales outgroups), which were used to analyze gene family evolution in *Armillaria* (Table S1:

Dataset1). This was extended into a phylogenetically more diverse Dataset2, which was used to

perform gene copy number analysis of wood-decay gene repertoires and comprised 131 fungal

species from both Basidiomycota and Ascomycota, including species with various lifestyles

such as white-rotters, brown-rotters, litter-decomposers, ECM fungi, pathogens, and species

with uncertain ecologies (Table S1: Dataset2). Dataset3 was used to identify horizontally

transferred genes and comprised 942 species, including species ranging from fungi, bacteria as

well as plants (Table S1: Dataset3). All the species used in these datasets are published and

publicly available sequences from JGI Mycocosm (except a few that were used with Author's

permission). Proteomes of all the species used in this manuscript were functionally annotated

by using InterPro (IPR) Scan (v.5.46-81.0) [98]. Secreted and small secreted (at least 300 amino

acids) proteins were identified as described previously [99].The luciferase cluster and its synteny

were identified in *Armillaria* species and 5 Physalacriaceae outgroups as described previously

[49]. Orthologous groups from these genomes were inferred using OrthoFinder (version 2.5.4)

[100,101] and synteny around the luciferase cluster was visualized based on ordering and orienting

scaffolds using the *Armillaria ostoyae* C18/9 genome and using genoPlotR [102] package. The

hispidin synthase and cytochrome P450 gene were previously missing and re-annotated on the

scaffold of NODE_104435 in *Armillaria mellea* DSM 3731 using predictions of *Armillaria mellea*

ELDO17 v1.0.

Genome evolution in *Armillaria* spp.

Reconstruction of genome wide gene family evolution was performed using Dataset1.

Predicted protein sequences were clustered into orthogroups using OrthoFinder (v2.3.1) [100,101]

with default settings. For the species tree, multiple sequence alignment for 81 single copy

orthogroups (SCOGs) were inferred using MAFFT (-auto) [103], followed by removal of gapped

regions from the alignments using TrimAl [104] with a gap threshold of 0.9. Trimmed alignments

were then concatenated into a supermatix using an in-house R-script. The species tree was

reconstructed in RAxML (v8.2.12) [105] with the option of rapid bootstrap analysis and search for

the best-scoring ML tree under the PROTGAMMAWAG model of protein evolution. The tree

was rooted using FigTree and is provided in Table S1.

We analyzed the genome-wide duplications and losses across gene families using the

COMPARE method [106] which uses reconciled gene trees to perform Dollo parsimony mapping

and ortholog coding to tell apart duplications from speciation events. To obtain the reconciled

gene trees for COMPARE, we first inferred multiple sequence alignments for the Orthofinder

orthogroups with at least 4 proteins using MAFFT (-auto) [103]. Aligned clusters were trimmed to

remove spurious regions using TrimAL [104] with -strict parameter. Clusters for which TrimAL

resulted in blank alignments (due to -strict parameter) were used in their non-trimmed form. In

total, 16,936 trimmed and 819 non-trimmed clusters were used in RAxML (v8.2.12) [105] for gene

tree inference, followed by Shimodaira-Hasegawa (sh)-like branch support calculation also in

RAxML under the PROTGAMMAWAG substitution model. The 17,755 gene trees with their SH-

like support values, along with the species tree were used for rerooting, followed by gene tree

species tree reconciliation in Notung (v2.9) run in batch mode with edge weight threshold set to

70. Reconciled gene trees were used to reconstruct the gene duplication/loss scenarios in

orthogroups along the species tree using the COMPARE pipeline (available at

https://github.com/laszlognagy/COMPARE). For each gene family, the number of gains, losses,

net gains (sum of gains and losses), and ancestral copy numbers were obtained and their

summary was mapped back onto the species tree. GO terms significantly enriched (p-values <

0.05) among the duplications at *Armillaria* MRCA were identified by topGO [42] using the weight01

algorithm and Fisher testing.

Analyses of CAZymes in *Armillaria* spp.

Dataset2 was used to analyze where Physalacriaceae (including *Armillaria*) fits in terms of

their wood-decay repertoires. Annotation of Carbohydrate Active enzymes (CAZymes) for the

required species were downloaded from JGI Mycocosm (species whose CAZymes were not

present in JGI Mycocosm were annotated using the CAZy annotation pipeline [107]. Based on

previously published reports, annotated CAZymes were further categorized based on their

putative plant cell wall degrading preferences (Table S3) into those acting on cellulose,

hemicellulose, pectin and lignin. We subjected the proteomes of these 131 species to

OrthoFinder (v2.3.1) [100,101] with default settings, giving us a total of 41,205 orthogroups. For

inferring the species tree, we generated SCOGs using a set of in-house R scripts which gave us

548 orthogroups in which the duplications were caused by gains at species levels (or terminal

gains). From these we omitted proteins showing less similarity based on amino acid distance

against the other members of the cluster. In this step we used at least 40% of all species for

each cluster. Finally, the resulting 514 SCOGs were concatenated (min 60 length of AA, and at

least 66 species) into one supermatrix and followed by species tree inference in IQ-Tree [108].

We generated phylogenetic PCA using the phyl.pca [109] function from Phytools [110] for

Physalacriaceae along with WR and LD fungi. We compare *Armillaria* and other

Physalacriaceae to only WR and LD fungi, because including brown rot and mycorrhizal

species, which have huge differences in gene content, reduced the resolution of the plots. We

provided the function with a gene copy number matrix normalized according to proteome sizes

for each substrate category and the above described ML species tree (subsetted to WR, LD and

Physalacriaceae species) as inputs. Independent contrasts were calculated under the Brownian

motion model and the parameter mode="cov". The substrate-wise gene copy numbers and their

resulting phylogenetic PCA loadings are provided in Table S3. We also plotted their proteome-

size normalized copy numbers into barplots for visualization of copy numbers across the

phylogeny (Figure S9, Figure S6, Figure S10, Figure S7, Figure S8).

We further investigated if Physalacriaceae members have CAZymes shared with

Ascomycota. For checking this hypothesis, we first identified the CAZy orthogroups from the

131 species, by selecting orthogroups with at least 50% of the proteins annotated as CAZymes,

and at least 5 proteins. This gave us 401 CAZy orthogroups which were used to perform

Fisher's exact test-based enrichment for identifying co-enriched CAZy families in

Physalacriaceae and Ascomycota with respect to WR and LD (Table S3).

Analyses of horizontally transferred genes

In order to check if Physalacriaceae species are more similar to Ascomycota than expected

by chance, which could either come from horizontal gene transfer or long retained ancestral

genes, we compiled the Dataset3. This comprised a taxonomically diverse dataset with a total of

942 species, ranging from 110 fungi from Basidiomycota (including 20 Physalacriaceae), 741

from Ascomycota, 26 from Mucoromycota, 10 from Zoopagomycota, 13 from Chytridiomycota

and 17 other early-diverging fungi as well as 15 bacterial and 10 plant species.

We identified candidate horizontally transferred (HT) genes using the Alien index (AI), for

which we ran MMseqs (easy-search, e-value 0.001) [111] using all Physalacriaceae proteomes

merged together as our query, against a database of proteomes of all the other species as our

subject. Using an in-house R script, we parsed the output to retain only the top hits (based on e-

values) for each taxonomic group. AI scores were calculated as $[\log_{10}$(best hit to species within

the group lineage $+ 1\times10^{-200}$) $- \log_{10}$(best hit to species outside the group lineage $+ 1\times10^{-200}$)]

[112,113].

Further, for phylogenetic validation of HT events, we used a subset of 329 species from

Dataset3 based on AI of top HT donors. In this case, we restricted our donor list to major HT

contributors only, *viz.* Ascomycota, followed by Mucoromycota and Zoopagomycota as

outgroups. The proteomes of these 329 species were subjected to OrthoFinder (v2.5.4) [100,101]

clustering with default settings. From these, we fished out the orthogroups with at least one AI-

based HT gene and also the orthogroups containing CAZymes. Further, we retained

orthogroups having at least one Physalacriaceae and one

Ascomycota/Mucoromycota/Zoopagomycotina species, resulting in a total of 675 orthogroups.

Proteins from these 675 orthogroups were aligned using MAFFT (-auto) [103] TrimAL (-strict)

[104]. Trimmed alignments were used to infer gene trees using IQ-Tree [108] with ultrafast bootstrap

(1000 replicates) under the WAG+G substitution model. Using an in-house R-script, we

identified potential HT events by extracting clades from the gene trees based on support values

(>70%) and taxon occupancy (receiver clade with >70% Physalacriacae species, donor clade

with >70% Ascomycota, and finally sister clade with >70% Ascomycota to ensure the direction

of gene transfer). Physalacriceae proteins from these putative HT clades, were used as a query

against the UniRef100 database [61] from the UniProt Reference Cluster using MMSeqs easy-

search, e-value 0.001) [111]. To remove the low sequence similarity matches and false hits, we

retained hits with percent identity and bidirectional coverage of more than 45%. We parsed the

filtered hits to retain only the top 100 hits (based on percent identity) for each query, and the

taxonomic category contributing predominantly among the top 100 hits was assigned as the

putative donor. Further, we classified the assigned donor into two categories: Type A, where the

top 100 hits were dominated and confidently associated with Ascomycota, and Type B, where

the top 100 hits were dominated by non-Agaricomycetes species, however ambiguously

distributed among different taxa (Table S4).

Live plant and stem invasion pathosystems

To understand gene expression in *Armillaria* and its host during the early stages of host

infection, we performed live *in planta* assays between *E. grandis* and *A. luteobubalina. A.*

luteobubalina was cultured onto half-strength modified Melin–Norkrans (MMN) media (pH 5.5;

1 g l^{-1} glucose) and grown for one month in the dark. *E. grandis* seeds were sterilized in 30%

hydrogen peroxide (H_2O_2, v/v) and germinated on 1% (w/v) water agar one month (25°C; 16 h

light cycle). Four weeks before contact with the fungus, seedlings were transferred onto half-

strength MMN medium ((pH 5.5; 1 g l^{-1} glucose) and grown at 22–30°C night/day temperature

with a 16 h light cycle. Once the *E. grandis* seedlings were two months old and the fungal

cultures had grown for one month, the plants were separated into one of three treatment

categories: (1) fungal-free controls whereby the seedlings were transferred onto new half-

strength MMN medium; (2) 'pre-symbiosis' which involved the transfer of seedlings onto new

half-strength MMN medium in indirect contact with fungal mycelium for 24 h by separating the

two organisms by a permeable cellophane membrane; (3) 'physical contact' seedlings were

transferred onto new half-strength MMN medium and then placed into direct contact with the

active growing edge of a fungal colony and then samples were harvested at 24 h, 48 h,

1 week, and 2 week post-contact. These plates were then closed using micropore tape to

allow for gas exchange with the external environment. Four biological replicates per treatment

and timepoint were generated and harvested at the described timepoint into liquid nitrogen and

stored at -80°C until RNA extraction.

For stem-invasion assays, high and low virulent isolates of conifer-specific *A. ostoyae* (C18 -

highly virulent, C2 - low virulent), and *A. borealis* (A6 - highly virulent, A4 - low virulent) [23,114]

were maintained in the dark on Petri dishes on RS medium (40g malt extract, 20g dextrose, 5g

bacto peptone, 19g agar / 1L) at 24°C and 4°C, respectively. Before the start of the experiment,

fresh cultures of all isolates were set up. The system for growing subcortical mycelial fans in the

laboratory consisted of plastic jars containing a layer of moistened and inoculated RSTO

medium [17]. After approximately 10 days of incubation in dark at 24°C, 10cm long, freshly cut

stem segments of Norway spruce were placed on top of this growing mycelial lawn, allowing

Armillaria to invade the stem segments. The timing and effectiveness of the advancing invasive

mycelial fans were followed by their arrival in small monitoring "windows" (1 x 1.5 cm) cut into

the bark halfway up to the top of the stems. The samples were harvested soon after the mycelial

front line appeared in the cutouts. *Armillaria* vegetative mycelia collected from the jars without

spruce stems were used as controls.

RNA isolation and sequencing

For *in planta* assays, live tissues, frozen samples were harvested and extracted using the

ISOLATE II Plant miRNA kit (Bioline, Sydney, Australia) as per the manufacturer's instructions.

Following extraction, the RNA samples were sequenced at the Beijing Genome Institute (BGI).

For the stem invasion assays, sections of the mycelial fan that were collected from under the

bark of infected colonized stems were frozen in liquid nitrogen and stored at -80°C. RNA was

isolated from the frozen samples using the RNeasy Plant Midi Kit (Qiagen Inc.) according to the

manufacturer's protocol. Prior to the RNA isolation, fungal tissues were homogenized with the

help of liquid nitrogen, mortar and pestle. RNA quantity was measured using Qubit

(ThermoFisher) according to the manufacturer's protocol. Biological triplicates were analyzed for

all sample types. The libraries for Illumina sequencing were prepared using NEBNext Ultra II

Directional RNA Library Prep Kit for Illumina (NEB, Ipswitch, MA, USA). Briefly, 100 ng RNA

was enriched using RiboCop rRNA Depletion Kits (Lexogen, Austria). Thereafter, the RNA was

fragmented, end prepped and adapter-ligated. Finally, the libraries were amplified according to

the manufacturer's instructions. The quality of the libraries was checked on Agilent 4200

TapeSation System using D1000 Screen Tape (Agilent Technologies, Palo Alto, CA, USA), the

quantity was measured on Qubit 3.0. Illumina sequencing was performed on the NovaSeq 6000

instrument (Illumina, San Diego, CA, USA) with 2 × 151 run configuration. Raw RNA-Seq reads

were aligned against the *A. ostoyae* (NCBI genome GCA_900157425.1 version 2) and *A.*

borealis (JGI: *Armillaria borealis* FPL87.14 v1.0) genomes using STAR v2.7 .5a [115]. After

alignment, the level of expression was estimated using RSEM v1.3.1 [116].

Analysis of expression data

1078

For the stem invasion assay, the estimated counts matrix was used for differential

expression analysis using Limma-Voom [117]. Prior to running the differential expression analysis,

counts were normalized using TMM (trimmed mean of M values) using edgeR v3.38.1 [118] and

lowly expressed genes (cpm ≤ 10) were filtered. Differential gene expression analysis for the *in-*

planta pathosystem data were carried out as our previous study [58].

In both analyses, genes with at least two-fold expression change and FDR value <=0.05

were considered significant. Multidimensional scaling ("plotMDS" function in edgeR) was applied

to visualize gene expression profiles. Expression heatmaps were generated by hierarchically

clustering based on average linkage of FPKM values with heatmap.2 function from gplots

package

To check reliability of both expression data, we used the superSeq package [83] which uses a

subsampling approach to simulate and predict the number of differentially expressed genes at

lower read depths and random sampling points from the original dataset. These subsampled

reads are then extrapolated to predict the relationship between statistical power and read depth.

Genes from the time-series *in planta* assay from both fungal and the plant side were

clustered into co-expression based modules using STEM (v1.3.11) [64]. GO terms significantly

enriched (p-values < 0.05) among each of the STEM modules were identified by topGO [42], using

the weight01 algorithm and Fisher testing.

Experimental validation of Pathogenicity-induced SSPs

1097

Aliquots of RNA extracted from the *in planta* pathosystem assay (6 timepoints, 24 samples)

were pooled and used for generation of full length cDNA using the Tetro cDNA synthesis kit

(Bioline) according to manufacturer's instructions and using only the oligo-dT primer. Two *A.*

luteobubalina HWK02 v1.0 SSP sequences were amplified using the KAPA HiFi Hotstart

Readymix (Roche) according the manufacturer's instructions using the following primers:

Armlut1_1165297(5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTYCARYTNYTNTTYGCNGC-3' and 5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACTCTTCTAGCAGCTTCACGTC-3') and

Armlut_1348401(5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTGTTCAGTTTCTTCCTCTTCTACC-3'

and 5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTGCTARTCNACRCANGTNGTNSWDATRTCNG-

3'). Successful amplification was verified using gel electrophoresis, products were gel purified

using the Wizard SV Gel and PCR Cleanup kit (Promega) and ligated into pDONR221-b1b4

(Invitrogen) using BP ligase (Invitrogen). Ligations were transformed into *E. coli* and positive

colonies were selected based on PCR screening. Positive bacterial colonies were grown

overnight in LB + kanamycin (50 mg/L) and plasmids purified using the PureLink HiPure

Plasmid Miniprep kit. Gene inserts were sequence verified using Sanger sequencing at the

Hawkesbury Institute for the Environment sequencing platform. Plasmids containing the proper

gene insert were used in an LR ligation with pBiFCt-2in1destination vector with an empty

cassette ligated into the b2b3 position. Similarly, as a control for *in planta* transformations, a

version of pBIFCt-2in1 with empty cassettes was generated as an empty vector control.

Screening procedures following were as per with pDONR221. Positive plasmids were purified

and transformed into *Agrobacterium tumefaciens* GV3101. Expression in *N. benthamiana* was

performed as per Plett et al., 2020 . Similarly, young leaves of 6 month old *E. grandis* saplings

grown at 25°C constant temperature (16hr light/8hr dark) were agroinfiltrated using the same

solutions as for *N. benthamiana.* As opposed to *N. benthamiana* leaves, *E. grandis* leaves were

more recalcitrant to infiltration without damaging the leaves, therefore the bacteria was only

introduced into a circle the size of the syringe bore. Both plant systems were left for seven days

and the leaves photographed at the site of infiltration. Leaves from three separate plants were

infiltrated for each plant model system and representative images presented.

A Estimated number of HT genes

Armnov1
Armlut1
Armfum1
Armmel1
Armme1_1
Armfus
Armga1
Armcep1
Armnab1
Armosto1
Armost1
Armbor1
Armtab1
Armect1
Guyne1
Flaro
Flave
Cylto1
Oudmuc1
Hymrad1

0   50   100   150   200

Alien-index based HT genes   Phylogenetically validated HT genes

B

Physalacriaceae
101 events
Other Basidiomycota
Lecanoromycetes   3
Eurotiomycetes   18
Dothideomycetes   28
Leotiomycetes   9
Sordariomycetes   28
Xylonomycetes
Pezizomycetes and Orbiliomycetes   1
Saccharomycotina   1
Taphrinomycotina
Other taxa   13

C

A. luteobubalina (Time-series)   *A. ostoyae* (Stem-invasion)   *A. borealis* (Stem-invasion)   *A. ostoyae* (Wood-decay)   *A. cepistipes* (Wood-decay)

Gene expression log2

Control 24hrs_memb 24hrs 48hrs 1week 2weeks

C18Control C18Fresh C2Control C2Fresh

A6Control A6Fresh A4Control A4Fresh

AROS_M AROS_NIM AROS_NIR AROS_R

ARCE_M ARCE_NIM ARCE_NIR ARCE_R

Phylogenetically validated HT genes   VT genes