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ARTICLE

Developing an experimental protocol to test *Armillaria*-specific genes for their potential role in pathogenicity and virulence

Boris Indic and György Sipos*

Functional Genomics and Bioinformatics Group, Faculty of Forestry, University of Sopron, H-9400 Sopron, Hungary

ABSTRACT Most *Armillaria* species are notorious fungal pathogens that cause substantial damage to woody plants in forestry and agriculture. Their pathogenic success is attributed to specialized gene families involved in host invasion and virulent activities in the plant tissues. This study aims to investigate the expression patterns of expanded genetically diversified gene families in two *Armillaria* species (*A. borealis* and *A. ostoyae*) using unique *Armillaria*-specific gene sets, involved in degrading monocyclic aromatics, previously identified through comparative genomics analysis of gene pools related to fungal bioremediation. By analyzing differential gene expression data from high- and low-virulent isolates grown under stem invasion conditions, comparing fresh, slowly decaying stems with autoclaved dead stems, several *Armillaria* genes were implicated as potential plant pathogenicity or virulence factors. In conclusion, systematic plant invasion experiments combined with differential gene expression profiling focusing on new gene variants specific to *Armillaria* species offer a reliable and feasible framework for predicting genes related to pathogenicity and distinguishing between pathogenicity genes and potential virulence effectors. **Acta Biol Szeged 70(1):40-46 (2026)**

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***Corresponding author**
E-mail: sipos.gyorgy@uni-sopron.hu

Introduction

Armillaria root rot, caused by the fungal pathogen *Armillaria* spp., is a devastating disease affecting many woody plants, inflicting substantial economic and ecological losses worldwide (Kedves et al. 2021). Due to their broad host range, environmental persistence, and the challenges associated with their detection and control, these fungi pose a significant threat particularly to weakened trees and unstable natural ecosystems in forestry and agriculture. Their pathogenicity is supported by a complex arsenal of virulence and pathogenicity factors encoded by specialized gene families (Sipos et al. 2017, 2018; Sahu et al. 2023). These gene families often encode secreted proteins, enzymes, and specific effectors that may play critical roles in host-pathogen interactions (Jha et al. 2023; Sahu et al. 2023).

The expression patterns and potential functions of these genes during different stages of vegetative growth and host interaction remain incompletely understood. Investigating these molecular mechanisms is crucial for developing effective disease prevention and management strategies.

Previous comparative genomic studies (Champramary et al. 2023) revealed unique gene sets in armillarioid species, emphasizing their genetic diversity and specialization in monocyclic aromatic compound degradation as a white root rot-causing agent participating in the degradation of lignin in the plant cell walls. The present study investigates the expression profiles of the gene repertoires in high- and low-virulent isolates of *A. ostoyae* and *A. borealis* and correlates their expression with potential roles in pathogenicity and virulence processes in host plants.

We hypothesize that pathogenicity and virulence-related gene expression is upregulated under plant tissue invasion conditions in response to various host-derived signals or substrates. To test this, *in vitro* stem invasion assays were established using fresh stems and autoclaved stems as dead wood tissue for invasive saprotrophic activities, and rich artificial media (RSTO) as non-invasive controls (Sahu et al. 2023).

This study aims to develop a procedure that combines gene expression profiling with functional diversification to identify potential virulence effectors in *Armillaria*. These findings advance our understanding of fungus-plant interactions and may aid in developing novel and targeted control strategies in the near future.

Materials and Methods

To predict potential pathogenicity and virulence factors, *in vitro* stem invasion assays were performed using high- and low-virulent isolates of two conifer-specific, primary

Table 1. The overall expression analysis of the genes of interest. The table shows the number of expressed genes detected in the mycelia growing under the bark of fresh (F) or autoclaved (A) Norway spruce stems and control mycelia grown on RSTO media (C), together with the total number of genes identified in the genomes of *A. ostoyae* and *A. borealis* encoding for the enzymes of interest. Three biological replicates were used for each of the groups, and genes were counted as expressed if their CPM (counts per million) value in at least two biological samples was higher than 1 CPM.

	Armillaria ostoyae (expressed/total in genome)						***Armillaria borealis*** (expressed/total in genome)					
	High-virulent			Low-virulent			High-virulent			Low-virulent		
Enzymes	F	A	C	F	A	C	F	A	C	F	A	C
NADPH2 dehydrogenase [EC:1.6.99.1]	19/20	18/20	19/20	16/20	11/20	16/20	13/14	14/14	10/14	14/14	12/14	10/14
Benzoate degradation												
Benzoate 4-monooxygenase [EC:1.14.14.92]	8/8	5/8	7/8	6/8	4/8	6/8	7/7	7/7	7/7	7/7	7/7	6/7
Monocyclic aromatics degradation												
Nitrilase [EC:3.5.5.1]	3/3	3/3	3/3	3/3	3/3	3/3	2/2	2/2	2/2	2/2	2/2	2/2
Homogentisate 1,2- dioxygenase [EC:1.13.11.5]	3/4	3/4	4/4	3/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
PAHs degradation												
Manganese peroxidase [EC:1.11.1.13]	1/7	0/7	4/7	0/7	0/7	6/7	6/8	7/8	7/8	8/8	7/8	7/8
Salicylate hydroxylase [EC:1.14.13.1]	7/8	6/8	8/8	6/8	2/8	7/8	9/9	9/9	9/9	9/9	9/9	9/9

pathogenic *Armillaria* species, *A. borealis* (low-virulent A4 - LWF ALP 8-2; high-virulent A6 - PBMD Schwanden) and *A. ostoyae* (low-virulent C2 - LWF LAU 10; high-virulent C18 - PBMD 3933) (Heinzelmann et al. 2017; Prospero et al. 2004). Freshly cut stem sections representing living, slowly decaying plant tissue ("Fresh") and autoclaved stems representing entirely dead wood ("Autoclaved") were inoculated with *Armillaria* isolates (plant-invasive conditions).

For the *in vitro* stem invasion assays, a nutrient-rich solid medium containing rice, sawdust, tomato, and orange (RSTO) (Sipos et al. 2017), which also already included the desired *Armillaria* inoculum, was prepared in 500 ml sterile plastic jars to grow vegetative mycelia. After three weeks, the stem segments were placed on the well-growing mycelial lawn to promote the growth of the invasive subcortical mycelial fans. The mycelia developing on substrate-rich RSTO media were considered for the non-invasive control condition.

All further experimental details regarding the *in vitro* stem invasion assays, RNA-Seq data analysis, and differential gene expression profiling are described in Sahu et al. (2023).

Gene expression data were analyzed with a focus on six key enzymes identified through the comparative genomic analysis of gene functions orthologous to biodegradative enzymes (Champramary et al. 2023): NADPH2 dehydrogenase [EC:1.6.99.1], benzoate 4-monooxygenase [EC:1.14. 14.92], nitrilase [EC:3.5.5.1], homogentizate 1,2-dioxygenase [EC:1.13.11.5], manganese peroxidase [EC:1.11.1.13] and salicylate hydroxylase [EC:1.14.13.1]. The phylogenetic trees and gene expression data were visualized using the Interactive Tree of Life (iTol) tool (Letunic and Bork 2021).

Results and Discussion

To test for possible functional specialization in pathogenic plant invasion in *Armillaria* species, we compared the expression profiles of genes involved in the degradation of monocyclic aromatics with those responsible for the degradation of polyaromatic compounds. The focus was on the NADPH2 dehydrogenase and benzoate 4-monooxygenase genes, as these two gene families were crucial for the separate clustering of *Armillaria* species in our original screen (Champramary et al. 2023).

Overall, the gene expression assays revealed the expression of all predicted genes of interest in both species, with significant differences based on the virulence of species/isolates and growth conditions (Table 1). Notably, NADPH2 dehydrogenase genes were more abundant in *A. ostoyae*, while the other gene families showed comparable numbers of orthologs in the two species. The expression of all genes showed no difference across all *A. borealis* isolates, regardless of the type of wood stem or their virulence, with all the genes present in the genome being expressed, while *A. ostoyae* produced different results between the isolates and the growth conditions (Table 1).

Regarding the NADPH2 dehydrogenase genes, while they were more abundant but expressed at lower levels in *A. ostoyae*, most orthologs of the less abundant genes were expressed at much higher levels in *A. borealis* (Fig. 1). Among the highly expressed genes in both high- and low-virulent *A. borealis* isolates, two genes that showed close phylogenetic relationship (Ambor1|1789714; Ambor1|1733777) were upregulated under plant-invasive conditions, indicating their potential role in pathogenicity (Fig. 1). Two other genes (Ambor1|140975; Ambor1|1893348) were expressed at high levels only in the highly

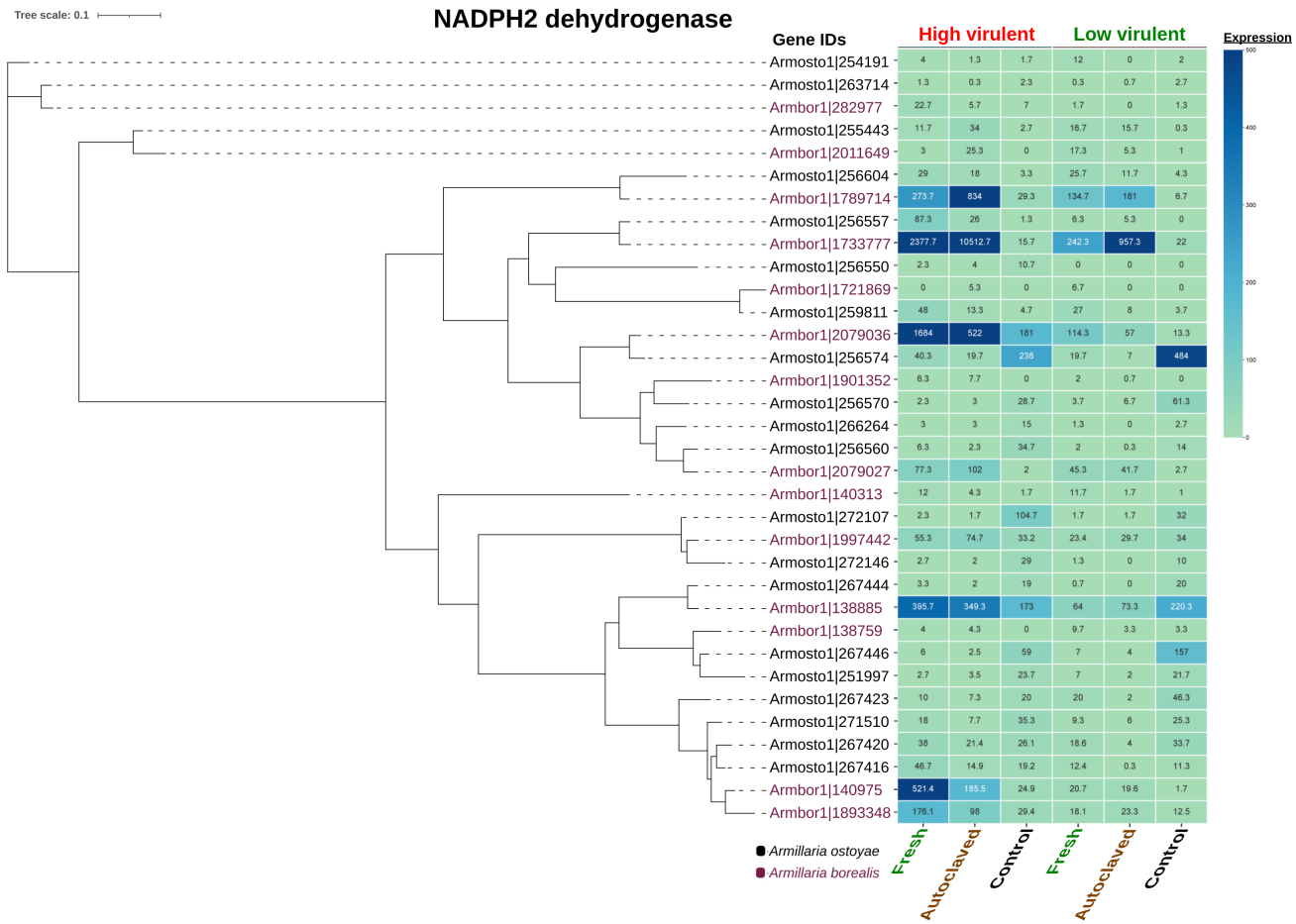

Figure 1. Gene expression analysis and phylogenetic diversity of the NADPH2 dehydrogenase genes. High- and low-virulent *A. ostoyae* and *A. borealis* isolates were used in the *in vitro* stem invasion assays. "Autoclaved" indicates mycelia isolated from under the bark of autoclaved wood stem segments, "Fresh" being the mycelia isolated from under the bark of non-autoclaved stem segments, and "Control" represents the mycelia grown on rich media.

virulent isolate when invading the plant tissues, especially under fresh stem conditions. The latter genes could be considered as possible candidates for specialized virulence effectors.

Benzoate 4-monooxygenase [EC 1.14.14.92], as an oxidoreductase enzyme, can convert benzoates to 4-hydroxybenzoate in a single step (Fuchs et al. 2011) and thus plays a critical role in benzoate metabolism. Six of the seven benzoate 4-monooxygenase genes were consistently expressed in *A. borealis* isolates (Fig. 2) regardless of the strain type and plant-invasive condition, with three genes (Armbor1|1888182, Armbor1|1721289 and Armbor1|1154540) appearing to be "pathogenic" as they were highly upregulated under stem invasive conditions. In addition, one *A. ostoyae* gene (Armosto1|267640) and one *A. borealis* gene (Armbor1|981169) showed expression patterns indicating that they could be classified as potential plant virulence effectors (Fig. 2).

Both *A. ostoyae* and *A. borealis* possess a richer genetic repertoire for the degradation of various monocyclic aromatic hydrocarbons than other fungi. This is evidenced by the abundance of genes encoding enzymes such as nitrilase [EC:3.5.5.1] and homogentisate 1,2-dioxygenase [EC:1.13.11.5] (Champramary et al., 2023).

Nitrilase hydrolyzes carbon-nitrogen triple bonds to produce amide products, ammonia, and carboxylic acid (Gunjal Aparna et al. 2021). Expression analysis of the nitrilase genes showed less pronounced but still significant signs of a potential plant virulence effector candidate in *A. ostoyae* (Armosto1|268415), and one *A. borealis* gene (Armbor1|1895917) exhibited an expression pattern indicative of a potential plant pathogenicity factor, as its expression was markedly higher in the highly virulent isolate in fresh and autoclaved stems (Fig. 3A).

Homogentisate 1,2-dioxygenase plays a crucial role in the degradation of homogentisate, a key intermediate in the degradation pathway for numerous aromatic compounds (Mori et al. 2016; Fernández-González et al. 2018).

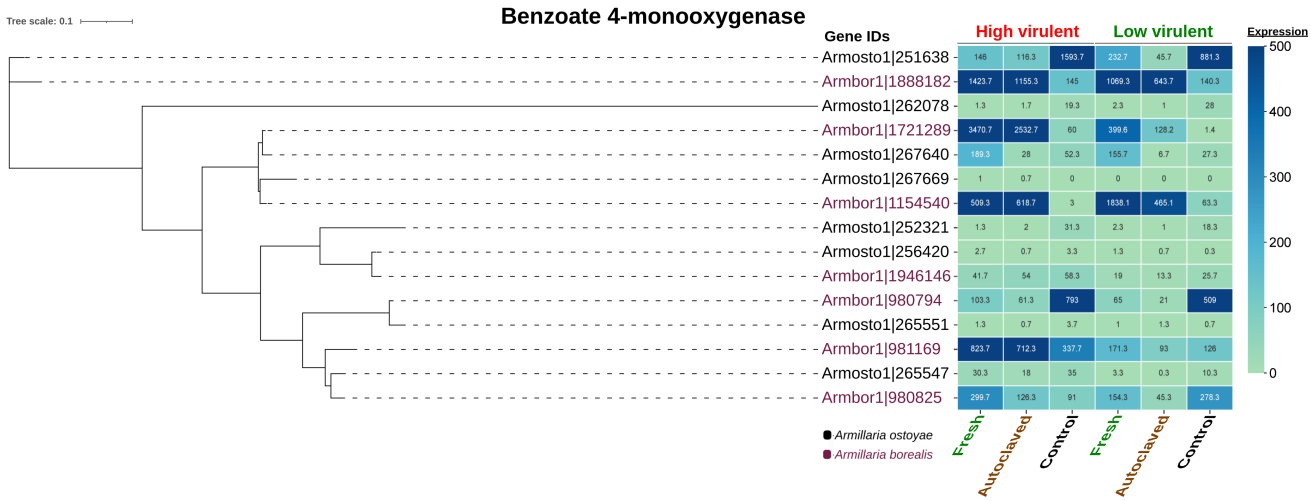

Figure 2. Gene expression analysis and phylogenetic diversity of the benzoate 4-monooxygenase genes. High- and low-virulent *A. ostoyae* and *A. borealis* isolates were used in the *in vitro* stem invasion assays. "Autoclaved" indicates mycelia isolated from under the bark of autoclaved wood stem segments, "Fresh" being the mycelia isolated from under the bark of non-autoclaved stem segments, and "Control" represents the mycelia grown on rich media.

This specialization suggests a potentially enhanced capacity of these *Armillaria* species to process a wider range of aromatic substrates. Among the genes present in *A. borealis*, the expression profiles of the Armbor1|1740943 and Armbor1|1909413 genes suggest that they could be potential plant pathogenicity factors, similar to the Armosto1|257280 gene expressed in *A. ostoyae* (Fig. 3B).

While genes associated with polycyclic aromatic hydrocarbon (PAH) degradation have been identified in armillarioids, their numbers were generally lower than in other basidiomycete species (Champramary et al. 2023). Among the enzymes involved in PAH degradation, salicylate hydroxylase [EC:1.14.13.1] and manganese peroxidase [EC:1.11.1.13] were more abundant in *A. ostoyae* and *A. borealis* compared to ascomycetes, but less abundant compared to other basidiomycetes. However, based on

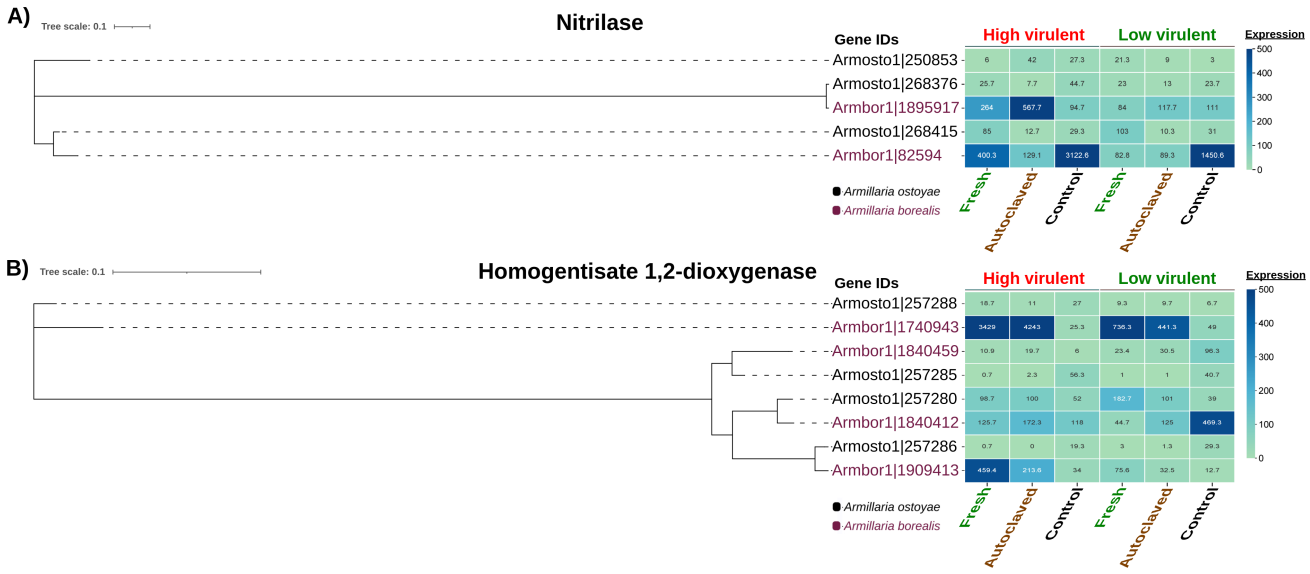

Figure 3. Gene expression analysis and phylogenetic diversity of the nitrilase and homogentisate 1,2-dioxygenase genes. High- and low-virulent *A. ostoyae* and *A. borealis* isolates were used in the *in vitro* stem invasion assays. "Autoclaved" indicates mycelia isolated from under the bark of autoclaved wood stem segments, "Fresh" being the mycelia isolated from under the bark of non-autoclaved stem segments, and "Control" represents the mycelia grown on rich media.

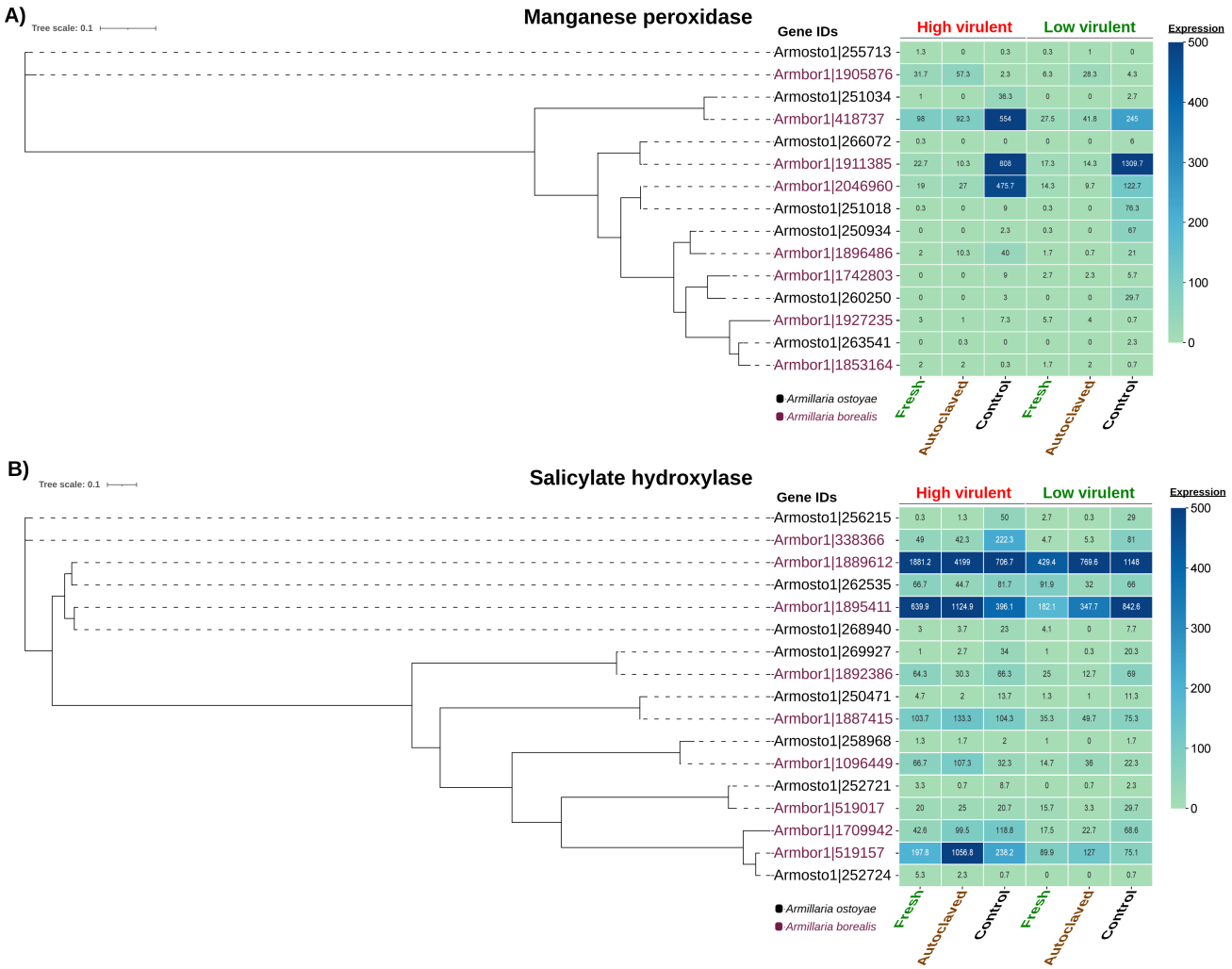

Figure 4. Gene expression analysis and phylogenetic diversity of the manganese peroxidase and salicylate hydroxylase genes. High- and low-virulent *A. ostoyae* and *A. borealis* isolates were used in the *in vitro* stem invasion assays. "Autoclaved" indicates mycelia isolated from under the bark of autoclaved wood stem segments, "Fresh" being the mycelia isolated from under the bark of non-autoclaved stem segments, and "Control" represents the mycelia grown on rich media.

their expression patterns, none of the genes indicated a potential contribution to pathogenicity or virulence, with their expression levels tending to increase or stay similar in noninvasive substrate-rich control media.

Conclusions

In this study, we describe a validation protocol using gene expression data from two necrotrophic *Armillaria* species to assess the potential influential role of genetic diversification within *Armillaria* gene families on pathogenicity and virulence.

The *Armillaria*-derived data sets were considered from a previously published genome-wide analysis (Champramary et al. 2023) that identified biodegradative enzymes with a potentially unique high capacity for monocyclic aromatic degradation in *Armillaria* species. Gene expression profiles of two conifer-specific species, *A. borealis* and *A. ostoyae* were examined in pairs of highly virulent and less virulent isolates from each species.

Isolates were tested in an *in-vitro* stem invasion system mimicking live and dead wood (fresh and autoclaved spruce stems), and nutrient-rich RSTO media as a noninvasive control. The assay provided an opportunity to identify selectively upregulated genes during plant invasion.

With this approach, we were able to reveal the possible complexity of the molecular mechanisms for seemingly homogeneous gene families at the genome level focusing on the correlation between the sequence changes of *Armillaria* gene sets and the emergence of potential plant

pathogenic and virulence impacts.

The positive correlation between *Armillaria*-specific functional gene diversification and the emergence of apparent pathogenic gene functions was further justified for examining white rot-related gene families involved in PAH degradation. None of the PAH-degrading enzyme genes appeared selectively invasive or pathogenic from the *Armillaria* isolates analyzed.

The similarities and differences observed in the strategies employed by *A. ostoyae* and *A. borealis* indicate only a limited number of identified factors. In addition, the functional overlap between closely related genes from different species is also limited. A finite number of enzymes were considered to test the hypothesis and the methodology, confirming that the suggested comparative approach can be successfully applied to identify potential pathogenicity and virulence factors. Thus, the method opens further possibilities for comparative studies of the entire *Armillaria* genome for functional specialization and for testing individual gene variations even beyond pathogenicity for any *Armillaria.* The consideration behind the test is suitable for investigating the pathogenicity and the virulence effectors of other plant pathogenic fungi in experiments transformed for other plant-fungus interaction systems.

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