SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Species-specific effects of the introduction of the *Aspergillus nidulans gfdB* gene in osmophilic *Aspergillus* species

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The PhD Defense takes place in Room F101 of the Life Sciences Laboratory building, University of Debrecen, at 13:00 on 12 June 2025.

INTRODUCTION

Our study focuses on the genetic and physiological responses of *Aspergillus* species to osmotic stress, which is a critical factor in optimizing industrial biotechnological processes. Understanding how genetic modifications can enhance stress tolerance in these fungi is essential for improving their applications in enzyme production and fermentation.

The *gfdB* gene, known for its role in environmental stress response in *Aspergillus nidulans*, was introduced into *Aspergillus wentii* to explore potential improvements in stress tolerance. This research aims to uncover the genetic and phenotypic changes that result from this modification. Techniques such as transcriptome analysis and quantitative PCR (qPCR) are employed to measure gene expression levels and assess physiological responses under various stress conditions, including high salinity (NaCl, sorbit) and osmotic pressure.

These observations are necessary because understanding the molecular mechanisms behind fungal stress tolerance can revolutionize industrial applications. Enhancing the stress tolerance of fungi used in enzyme production can lead to more efficient and cost-effective manufacturing processes. Insights gained from this

study could also provide a broader understanding of fungal biology and stress adaptation mechanisms.

The host's native genetic regulatory network may influence the expression and efficacy of the introduced gene. Additional genetic targets could be modified to further enhance stress tolerance. Hovewer the broader implications of these genetic modifications on the overall fitness and industrial utility of the modified fungi remain to be explored.

Overall the main scope of the study is the presentation of the examined *Aspergillus* species, the aspect of osmoadaptation, the investigation of the role of the *Aspergillus nidulans gfdB* gene, with stress tolerance and transcription methods.

AIM

At the beginning of our work, we aimed to answer the following questions: (1) Can the stress tolerance of the industrially relevant *A. wentii* be improved through targeted genetic modifications involving the *gfdB* gene? (2) Can gene dosage changes involving the *gfdB* gene lead to the emergence of new phenotypes (e.g., osmotolerance) in the filamentous fungus *A. wentii*? (3) Can the phenotype of *A. nidulans* be reconstructed in the osmotolerant *A. wentii*, which is evolutionarily more distant, through genetic modification involving the *gfdB* gene? (4) Can we gain insights into the modularization processes underlying the osmotolerant phenotype through a transcriptomic approach?

To answer these questions, we conducted the following investigations:

1. We conducted stress sensitivity tests on *A. wentii* CBS141173 mutant and wild-type strains transformed with the *An-gfdB* gene, in the presence and absence of osmotic stress-, cell wall integrity-, heavy metal-, and oxidative stress inducing agents.

2. We performed comparative stress sensitivity tests using the *A. glaucus* CBS516.65 mutant and wild-type strains, and compared them with the osmotolerant *A. wentii* CBS141173 mutant and wild-type strains.

3. We applied a multidimensional approach for stress based positioning of *Aspergillus* spp., involving the analysis of an additional 17 *Aspergillus* species.

4. We conducted transcriptional studies in the presence of osmotic stress inducing agents, to compare the expression of the *gfdB* gene in *A. nidulans* THS30 and *A. wentii* CBS141173 wild-type and mutant strains.

MATERIALS AND METHODS

Strains and Culture Conditions

In this study we utilized the following *Aspergillus* strains: *A. nidulans* THS30.3, *A. nidulans ΔgfdB*, *A. glaucus* CBS516.65, *A. glaucus* CBS516.65 *'c gfdB1-2*, *A. wentii* CBS141173, and *A. wentii* CBS141173 *'c gfdB1-3*. Conidia of *A. wentii* and *A. glaucus* were produced on malt extract agar (MEA) with NaCl supplementation for *A. glaucus*. *A. nidulans* strains were sporulated on nitrate minimal medium (NMM). Conidiospores were suspended in sterile water, filtered, and spore counts determined using a hemocytometer.

For transcriptional analysis all strains were maintained on Barratt minimal agar plates at 25°C. Conidia collected on day 6 were used to inoculate submerged cultures. Cultures were inoculated with $1x10^8$conidia in 100 ml of Barratt minimal broth in 500 ml Erlenmeyer flasks and incubated at 25°C on a rotary shaker at 220 rpm (approximately 3.7 Hz). *A. nidulans* mycelium was collected after 36 hours (THS30) and 38 hours (*ΔgfdB*), while *A. wentii* mycelium was collected after 65 hours for both CBS141173 and *'c gfdB strains*. The mycelium was washed and transferred to fresh Barratt minimal broth or broth supplemented with 2 M sorbitol, 1 M

NaCl, and 1 M NaCl + 2 M sorbitol. Cultures were further incubated at 25°C and 220 rpm, with samples taken after 0.5 hours (for RNA isolation) or 10 hours (for DCM determination).

Generation of Transgenic Strains

The pAN7.1 plasmid containing the hygromycin B resistance gene was used to transform *A. nidulans* with the *gfdB* gene. Protoplasts were prepared from exponential phase cultures using *Trichoderma harzianum* lysing enzyme and transformed with the plasmid using a PEG-based method. Transgenic strains were confirmed via PCR.

Genotype Verification

qPCR was employed to determine the copy number of the *gfdB* gene in *A. wentii* transgenic strains. Serial dilutions of genomic DNA were used with Fast SYBR® Green master mix on a LightCycler® 480 instrument. PCR cycles were conducted to ensure the presence of the *gfdB* gene.

Stress Adaptation Studies

In this study we examined the stress sensitivity and viability of the listed strains in the Strains and Culture Conditions section.

Stress sensitivity was assessed using large-scale agar plate assays. Freshly collected spores (1×10^5) were spot-inoculated on NMM agar plates and incubated at 25°C for 5 and 10 days. Various stress-inducing agents were added to the NMM agar, including Congo red, tert-butyl hydroperoxide (*t*BOOH), hydrogen peroxide (H_2O_2), menadione sodium bisulfite (MSB), diamide, $CdCl_2$, sorbitol, and NaCl. Colony diameters were measured to evaluate stress sensitivity.

Comparative Stress Tolerance Analysis

A comparative stress tolerance analysis was performed using cluster analysis and multidimensional scaling (MDS) for *Aspergillus* strains, including data from *A. wentii* CBS141173, *A. wentii* CBS141173 *'c gfdB1*, *A. glaucus* CBS516.65, *A. glaucus* CBS516.65 *'c gfdB1*, *A. nidulans* THS30.3, and *A. nidulans ΔgfdB*. Data from the Fungal Stress Database (FSD) were also included for 15 additional *Aspergillus* species. MIC_{50} values were determined for H_2O_2, MSB, and $CdCl_2$ after 5 and 10 days of incubation at 25°C. Relative growth values for NaCl, Congo red, and sorbitol were used for cluster analyses performed with the R software. Standardized values and Euclidean distances between strains were calculated, and cladograms and MDS diagrams were created to visualize stress sensitivity differences and similarities.

Transcriptional Analysis

RT-qPCR assays were carried out employing an Xceed SG 1-step 2× Mix Lo-ROX qPCR Kit (Institute of Applied Biotechnologies, Prague, Czech Republic), following the manufacturer's protocol. Relative transcription levels were characterized with the ΔCP (difference between the crossing point of the reference and the target gene within a sample) values using AN6542 (*actA*; *γ-actin*) for the *A. nidulans* samples and Aspwe1_0167845 (putative translation elongation factor EF-3) for the *A. wentii* samples as reference genes.

Total RNA was isolated from lyophilized mycelium using the method described by Chomczynski (1993) from four different culture conditions (untreated, 2 M sorbitol, 1 M NaCl, and 2 M sorbitol + 1 M NaCl) for each strain (*A. nidulans* THS30 and *ΔgfdB*, *A. wentii* CBS141173 and *'c gfdB*), with three biological replicates (total of 48 samples). RNA sequencing, from library preparation to fastq.gz file generation, was conducted at the Genomic Medicine and Bioinformatics Core Facility, Department of Biochemistry and Molecular Biology, University of Debrecen. Single-read 75 bp Illumina sequencing yielded 12–60 million reads per sample. HISAT2 (version 2.1.0) was used for reference genome alignment

(84-96% alignment rate) and BAM file generation, while featureCounts (version 2.0.0) was used for read counting.

Differential expression analysis was performed using DESeq2 (version 1.36.0), with RPKM values calculated using the edgeR package. Principal component analysis was conducted with the "prcomp" function. For the *gfdB* gene (absent in the *A. wentii* CBS141173 genome), reads corresponding to the *A. nidulans gfdB* sequence were counted using BBmap (version 39.03) with "perfectmode" settings, and these counts were used to calculate RPKM values. The differences in RPKM values calculated by BBmap and edgeR for the *A. nidulans* THS30 strain were less than 10%.

Transcriptome Data Evaluation

Differentially expressed genes (DEGs) were identified with a $\log_2$ fold change ($\log_2$FC) threshold, considering genes with a corrected p-value < 0.05. DESeq2 was used to calculate $\log_2$FC values, using untreated cultures as a reference. Gene set enrichment analyses were performed using ShinyGO and FungiDB platforms, with a focus on GO and KEGG pathways containing three or more genes and a corrected p-value < 0.05. DEGs were also analyzed

using Pearson correlation coefficients and Venn analysis for transcriptional changes.

Genes related to osmotic stress in *A. nidulans* were identified from literature and databases, and their orthologs in *A. wentii* were collected from FungiDB. Enrichment of these genes was analyzed using Fisher's test in the R software.

Statistical Methods

The effects of stress treatments and genetic modifications on the *A. wentii* CBS141173, *A. wentii* CBS141173 *'c gfdB1*, *'c gfdB2*, and *'c gfdB3*, as well as the *A. glaucus* CBS516.65 and *A. glaucus* CBS516.65 *'c gfdB1* and *'c gfdB2* strains were analyzed using two-factor analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences in average colony diameter values were considered significant if the adjusted p-value was less than 0.05 (Király et al. 2020a,b).

THE SUMMARY OF NEW SCIENTIFIC RESULTS

1. Introduction of *An-gfdB* in *A. wentii*

1.1. **Osmophilic Nature Maintained**: We demonstrated that the introduction of the *gfdB* gene from *Aspergillus nidulans* into *Aspergillus wentii* did not eliminate its osmophilic nature. This suggests that the inherent osmophilic characteristics of *A. wentii* are robust and not easily overridden by the introduction of a single gene. In the case of *A. wentii*, the osmophily decreased compared to *A. glaucus*, indicating that partial phenotype complementation was achieved, which supports the hypothesis proposed by de Vries in his 2017 publication.

1.2. **Reduced Growth**: Expression of *An-gfdB* in *A. wentii* led to a significant reduction in growth under high osmolarity conditions. This indicates that while *A. wentii* retains its osmophilic properties, the presence of *An-gfdB* interferes with its optimal growth, possibly due to metabolic imbalances or misregulation of stress response pathways.

1.3. **Transcriptomic Alterations**: Detailed transcriptomic analysis revealed that *A. nidulans* typically responds to hyperosmotic stress by upregulating genes associated with trehalose and glycerol

metabolism, along with the high-osmolarity glycerol (HOG) pathway genes. This canonical stress response was notably absent in *A. wentii*, indicating a fundamental difference in how these species manage osmotic stress.

1.4. **Impact of Gene Deletion**: The deletion of *gfdB* in *A. nidulans* caused only minimal changes in the transcriptome, suggesting that this species has a highly flexible glycerol metabolism that can compensate for the loss of the GfdB enzyme. This metabolic flexibility is likely a key factor in the osmotolerance of *A. nidulans*.

1.5. **Species-Specific Responses**: The introduction of *An-gfdB* in *A. wentii* led to more pronounced transcriptomic changes compared to the effects of *gfdB* deletion in *A. nidulans*. This underscores the species-specific nature of osmoadaptation mechanisms and the complexity of metabolic networks involved in stress responses.

1.6. **Metabolic Flexibility**: The presence of two differently regulated *gfd* genes in *A. nidulans* appears to confer a significant advantage for surviving in environments with fluctuating osmolarity. In contrast, *A. wentii*, which naturally reproduces in environments with consistently high osmolarity, contains only a single *gfd* gene, a *gfdA* ortholog, which indicates different evolutionary adaptation strategies for coping with osmotic stress.

2. Disruption of Osmoadaptation in *A. wentii* by *An-gfdB*

2.1. **Disruption of Natural Osmoadaptation**: The expression of *An-gfdB* in *A. wentii* disrupted its natural osmoadaptation mechanisms. This disruption was evidenced by inhibited growth and significant alterations in gene expression patterns, indicating that *An-gfdB* interferes with the finely tuned osmophilic adaptations of *A. wentii*.

2.2. **Gene Expression Misregulation**: RNA sequencing showed that the introduction of *An-gfdB* in *A. wentii* led to the misregulation of key osmoadaptive genes. These genes, which are typically stable and regulated in *A. nidulans*, became dysregulated in *A. wentii*, highlighting the incompatibility of *An-gfdB* with the osmophilic genetic framework of *A. wentii*.

2.3. **Different Stress Responses**: Our study highlighted significant differences in stress response mechanisms between osmotolerant species like *A. nidulans* and osmophilic species like *A. wentii*. Based on our results, the presence of the two *gfd* genes is beneficial for osmotolerant *Aspergillus* species that have to adapt to environments with fluctuating osmotic pressure, whereas in osmophilic species that are continuously exposed to high osmolarity, these genes are under selective pressure.

2.4. **Implications for Genetic Engineering**: Understanding the species-specific effects of *gfdB* can inform strategies for engineering fungal strains with desired osmotolerance traits for industrial applications. The findings suggest that genetic modifications need to consider the intrinsic stress adaptation mechanisms of the target species to avoid unintended phenotypes.

2.5. **Significant Transcriptomic Impact**: The introduction of *An-gfdB* caused significant transcriptomic changes in *A. wentii*, far beyond what was observed with *gfdB* deletion in *A. nidulans*. This indicates that *A. wentii*'s osmophilic traits are highly specialized and sensitive to genetic alterations that interfere with its established metabolic and regulatory networks.

SUMMARY

The efficiency of industrial applications of fungi largely depends on their stress tolerance, the enhancement of which could revolutionize biotechnological and agricultural processes. Investigating the stress induced by intracellularly compatible solutes such as glycerol, mannitol, erythritol, and arabitol (Sánchez-Fresneda et al. 2013; de Lima et al. 2015; Király et al. 2020a) in submerged *Aspergillus* cultures could offer a way to increase sugar alcohol yields in industrial fermentation processes—a field that has primarily focused on osmotolerant/osmophilic yeasts until now (Moon et al. 2010; Yang et al. 2021; Erian and Sauer, 2022; Yaakoub et al. 2022).

Our experiments revealed that the insertion of the *An-gfdB* gene with its own promoter and terminator sequences into the osmophilic *A. wentii* did not enhance the strain's stress tolerance. Therefore, targeted strategies to improve this capability must consider potential species-specific responses. We also hypothesize that the *A. nidulans gfdB* gene, when paired with its own promoter and integrated into the genomes of *A. glaucus* and *A. wentii*, interacts with various transcription factors, resulting in altered stress responses and promoter preferences (Wohlbach et al. 2009). This contributes to the phenotypic differences observed in the *A. glaucus*

and *A. wentii c' gfdB* strains (Király et al. 2020b). This implies that we need to identify not only genes that enhance stress tolerance but also regulatory elements that ensure the effective and safe functioning of these genes within fungal genomes.

Furthermore, MDS analysis showed that inserting this gene with its own regulatory elements did not bring the two osmophilic species (*A. wentii*, *A. glaucus*) evolutionarily closer to the osmotolerant *A. nidulans*. Transcriptome analysis suggests that the absence of the *gfdB* gene in *A. wentii* is not the cause of osmophilia but rather a consequence of adaptation to high osmolarity environments.

In summary, our study highlights the complex interplay between genetic modifications and species-specific physiological responses in fungi. By uncovering the molecular mechanisms underlying osmoadaptation, these findings pave the way for developing targeted strategies to enhance stress tolerance and optimize biotechnological processes. Future research in this area holds great promise for advancing our understanding of microbial biology and harnessing the full potential of fungi in biotechnological applications.

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List of publications related to the dissertation

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2. **Bodnár, V.**, Király, A., Orosz, E., Miskei, M., Emri, T., Karányi, Z., Leiter, É., De Vries, R. P., Pócsi, I.: Species-specific effects of the introduction of Aspergillus nidulans gfdB in osmophilic aspergilli.
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List of presentations and posters

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Bodnár, V., Pákozdi, K., Király, A., Póliska, S., Antal, K., Leiter, É., Pócsi, I., Emri, T. (2023). Osmotic stress elicited gene expression changes in *Aspergillus wentii* wild-type and *'c gfdb* and *Aspergillus nidulans* wild-type and *gfdb* mutant strains. Acta Microbiologica et Immunologica Hungarica, 70, Supplement 1, p. 58.

Bodnár, V., Király, A., Orosz, E., Emri, T., Karányi, Z., Leiter, É., de Vries, R. P., Pócsi, I. (2022). The physiological effects of the supplementation and expression of *Aspergillus nidulans gfdB* in other *Aspergillus* spp. are species-specific. In: A Magyar Mikrobiológiai Társaság 2022. évi Nagygyűlése és a XV. Fermentációs Kollokvium: Absztraktfüzet. (2022), p. 10, 1 p.